

**Sialate O-Acetylation and De-O-Acetylation
in Human Colonic Mucosa**

Dissertation

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**Sialate *O*-Acetylation and De-*O*-Acetylation
in Human Colonic Mucosa**

Thesis submitted for the degree of Doctor of Philosophy

by

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Statement of Originality

I declare that this thesis has not been submitted in any form for another degree or diploma at any university. Results discussed in this thesis are my own work, and information derived from the literature or unpublished work of others has been acknowledged in the text and a list of references provided.

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Abbreviations

AcCoA	acetyl-coenzyme A
BSA	bovine serum albumin
BSM	bovine submandibular gland
BIAL reagent	orcinol-Fe ³⁺ -HCl
CHAPS	3-((3-cholamidopropyl)-di-methylammonio)-1-propane-sulfonate
CMC	critical micellization concentration
CMP	cytidine 5'-monophosphate
CoA	coenzyme A
Cse	cytosolic sialate- <i>O</i> -acetyltransferase
Cys	cysteine
DFP	diisopropylfluorophosphate
DEPC	diethylpyrocarbonate
DIDS	4,4'-diisothiocyanostilbene-2,2'-disulfonic acid
DMB	1,2-diamino-4,5-methylenedioxybenzene
DTT	dithiothreitol
EBNS	edible bird nest substance
EDTA	ethylenediaminetetraacetic acid
EI	electronic impact
FAB-MS	fast atom bombardment-mass spectrometry
Gal	galactose
GalNAc	<i>N</i> -acetylgalactosamine
GlcNAc-6-P	<i>N</i> -acetylgalactosamine-6-phosphate
GD ₃	disialoganglioside
Glc	glucose
GlcNAc	<i>N</i> -acetylglucosamine
GlcNH ₂ -6-P	<i>N</i> -acetylglucosamine-6-phosphate
GLC-MS	gas-liquid chromatography-mass spectrometry
HCM	human colonic mucin
His	histidine

HPLC	high-performance liquid chromatography
HPTLC	high-performance thin-layer chromatography
Kdn	3-deoxy-D- <i>glycero</i> -D- <i>galacto</i> -non-2-ulopyranosonic acid
KI-8110	5-fluoro-2',3'-isopropylidene-5'- <i>O</i> -(4- <i>N</i> -acetyl-2,4-deoxy-3,6,7,8-tetra- <i>O</i> -acetyl-1-methoxycarbonyl-D- <i>glycero</i> - α -D-galactooctapyranosyl)uridine
K_i	dissociation constant of inhibitor
K_M	Michaelis-Menten constant
Lse	lysosomal sialate- <i>O</i> -acetyltransferase
LUBROL Px	polyoxyethylene 9 lauryl ether
MAG	myelin-associated glycoprotein
ManNAc	<i>N</i> -acetyl-D-mannosamine
MS	mass spectrometry
MU	methylumbelliferon
MU-Ac	4-methylumbelliferyl acetate
Neu5Ac	<i>N</i> -acetylneuraminic acid
Neu5,9Ac ₂	5- <i>N</i> -acetyl-9- <i>O</i> -acetylneuraminic acid
Neu5,7Ac ₂	5- <i>N</i> -acetyl-7- <i>O</i> -acetylneuraminic acid
Neu2en5Ac	5- <i>N</i> -acetyl-2-deoxy-2,3-didehydro-neuraminic acid
Neu5Gc	<i>N</i> -glycolylneuraminic acid
NMR	nuclear magnetic resonance spectrometry
Nonidet P40	nonaethylene glycol octylphenyl ether
OAE	sialate- <i>O</i> -acetyltransferase
7(9)-OAT	acetyl-CoA:sialate-7(9)- <i>O</i> -acetyltransferase
4-OAT	acetyl-CoA:sialate-4- <i>O</i> -acetyltransferase
PBS	phosphate-buffered saline
PCA	perchloroacetic acid
<i>p</i> -CMB	<i>p</i> -chloromercuribenzoate
PK buffer	50 mM phosphate buffer, pH 7.0
ST	sialyltransferase
TCA	trichloroacetic acid

TLC	thin-layer chromatography
Triton X-100	nonaethylene glycol octylphenol ether
Tris	tris(hydroxymethyl)aminomethane
Trp	tryptophane
Try	tyrosine
Vmax	maximal velocity (or rate)

1. Introduction

1.1 Occurrence of Sialic Acids

1.1.1 Structural Basis for Diversity in Sialic Acids

Since the first discovery of sialic acids at the end of the 1930s, more than 50 naturally occurring derivatives have been found [1;2]. Structurally, sialic acids are a family of acidic nine-carbon sugars, originally described as derivatives of 5-amino-3,5-dideoxy-D-*glycero*-D-*galacto*-non-2-ulosonic acid (neuraminic acid; Neu). The most widespread form is 5-acetamido-3,5-dideoxy-D-*glycero*-D-*galacto*-non-2-ulopyranosonic acid (*N*-acetylneuraminic acid; Neu5Ac), which is believed to be the biosynthetic precursor for all other members of the family. Hydroxylation of the *N*-acetyl group leads to 5-hydroxyacetamido-3,5-dideoxy-D-*glycero*-D-*galacto*-non-2-ulopyranosonic acid (*N*-glycolyneuraminic acid; Neu5Gc), which occurs frequently in many animal species, but has been found in humans only in the case of particular cancer [2;3]. The 5-amino group can be replaced by a hydroxyl group, yielding 3-deoxy-D-*glycero*-D-*galacto*-non-2-ulopyranosonic acid (Kdn). Further structural variation of sialic acids arises from substitutions of one or more of the hydroxyl groups of Neu5Ac, Neu5Gc or Kdn with acetyl, methyl, lactyl, phosphate or sulphate groups, or introduction of a double bond between C-2 and C-3 [2;4].

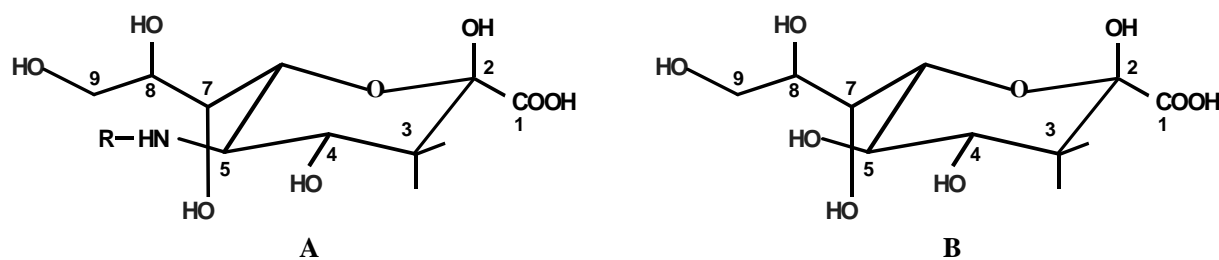


Figure 1-1 Structures of Sialic Acids. A: Neuraminic acid (Neu, R=H-); *N*-acetylneuraminic acid (Neu5Ac, R=CH₃CO-); *N*-glycolyneuraminic acid (Neu5Gc, R=HOCH₂CO-). B: 3-deoxy-D-*glycero*-D-*galacto*-non-2-ulopyranosonic acid (Kdn).

1.1.2 Distribution of Sialic Acids in Nature

Sialic acids have mainly been found in the animal kingdom, from the echinoderms to humans [1;2]. It has been reported that sialic acids are also present in some viruses, various bacteria, protozoa, and pathogenic fungi [1;2;4].

Generally, sialic acids are localized at the terminal position of a glycan chain on glycoproteins and glycolipids as well as oligosaccharides, and are either α 2,3- or α 2,6-linked to D-galactose (Gal), α 2,3- or α 2,6-linked to *N*-acetyl-D-galactosamine (GalNAc), α 2,6-linked to *N*-acetyl-D-glucosamine (GlcNAc), or α 2,8-linked to other sialic acids [2].

1.1.3 *O*-Acetylated Sialic Acids and the Spontaneous Migration of *O*-Acetyl Groups

O-Acetylation is one of the most common modifications that occur on sialic acids. It can occur either at position 4 of the pyranose ring [5;6], or at position 7, 8 and 9 of the glycerol side chain of sialic acids [2;4;7;8]. One interesting chemical property of *O*-acetylated sialic acids is that the *O*-acetyl group at position 7- or 8- can spontaneously migrate to an unsubstituted hydroxyl at position 9 [9;10]. The migration rate of 8-*O*-acetyl group is too fast to isolate the compound in a stable state. In contrast, the migration half-life of free Neu5,7Ac₂ is 4-8 h at physiological conditions (37 °C, pH 7.0) [9]. However, the migration rate is considerably slower at 37 °C, pH 6.5. Although it has not been accurately determined, the migration of *O*-acetyl groups on glycosidically linked sialic acids is assumed to be similar.

The migration of *O*-acetyl groups might be significant for the biosynthesis and biological properties of *O*-acetylated sialic acids. *In vivo*, it is possible that *O*-acetylated sialic acids on specific molecules are first expressed at the cell surface as Neu5,7Ac₂, whose 7-*O*-acetyl groups could subsequently migrate to the 9-position over time. As a result, the recognition or biological properties of the underlying molecules are altered due to a change in structure [4;7].

1.1.4 Biological Roles of *O*-Acetylated Sialic Acids

The distribution of *O*-acetylated sialic acids was first thought to be a species-specific determinant, since Neu5,9Ac₂ was found in bovine submandibular gland mucin, and Neu5,4Ac₂ in equine submandibular gland mucin [2;4;7]. However, with the improvement in analytical techniques, *O*-acetylated sialic acids have been found nearly in all higher animals and certain bacteria, suggesting an important biological role of these molecules. We are just at

the beginning of understanding this aspect, however, more and more evidences suggest that *O*-acetylated sialic acids be involved into many physiological and pathological processes (Table 1-1).

Table 1-1 **Physiological Functions of *O*-Acetylated Sialic Acids.**

Effect	References
Modification of the physicochemical properties of glycoconjugates.	[8;11]
Hindrance of the action of sialidase, <i>trans</i> -sialidase, sialate-pyruvate-lyase <i>etc.</i>	[2;8]
Targets for a variety of microbial and viral proteins such as influenza C virus, mouse hepatitis virus, coronavirus, encephalomyelitis virus.	[12-14]
Masking the recognition sites, e.g., for influenza A and B viruses, <i>Plasmodium falciparum</i> , sialoadhesin, CD22 and MAG.	[15;16]
As the specific ligands playing important roles in cell and molecule recognition phenomena, for example, as the epitopes for recognition by antibodies and lectins.	[2;8;11]
Tumor associated antigens, e.g., human melanoma (9- <i>O</i> -Ac-GD ₃), basalioma (9- <i>O</i> -Ac-GD ₃).	[2;7;17;18]

1.2 **Metabolism of Sialic Acids**

The main pathway of sialic acids metabolism has been clarified. Figure 1-2 summarizes the basic steps in the eukaryotic cells, and the subcellular localizations of the main enzymes involved [2]. Free Neu5Ac is synthesized in the cytosol, which is activated to CMP-Neu5Ac in the nucleus. *En route*, CMP-Neu5Ac can be hydroxylated to yield CMP-Neu5Gc by the CMP-Neu5Ac hydroxylase. In the Golgi apparatus, sialic acids are transferred from CMP-sialic acids to glycoconjugates by sialyltransferases. The sialic acids on glycoconjugates can be further modified.

The degradation of un-substituted sialic acids begins with the hydrolysis of sialic acids by sialidase that is located in the lysosome. In some cases, modification present on sialic acids

hinders the action of sialidases, thus, other enzymes are required to remove these modifications to facilitate the action of sialidases. Free sialic acids are transported from the lysosomal compartment to the cytosol, and are re-utilized or degraded by a cytosolic sialate-pyruvate-lyase.

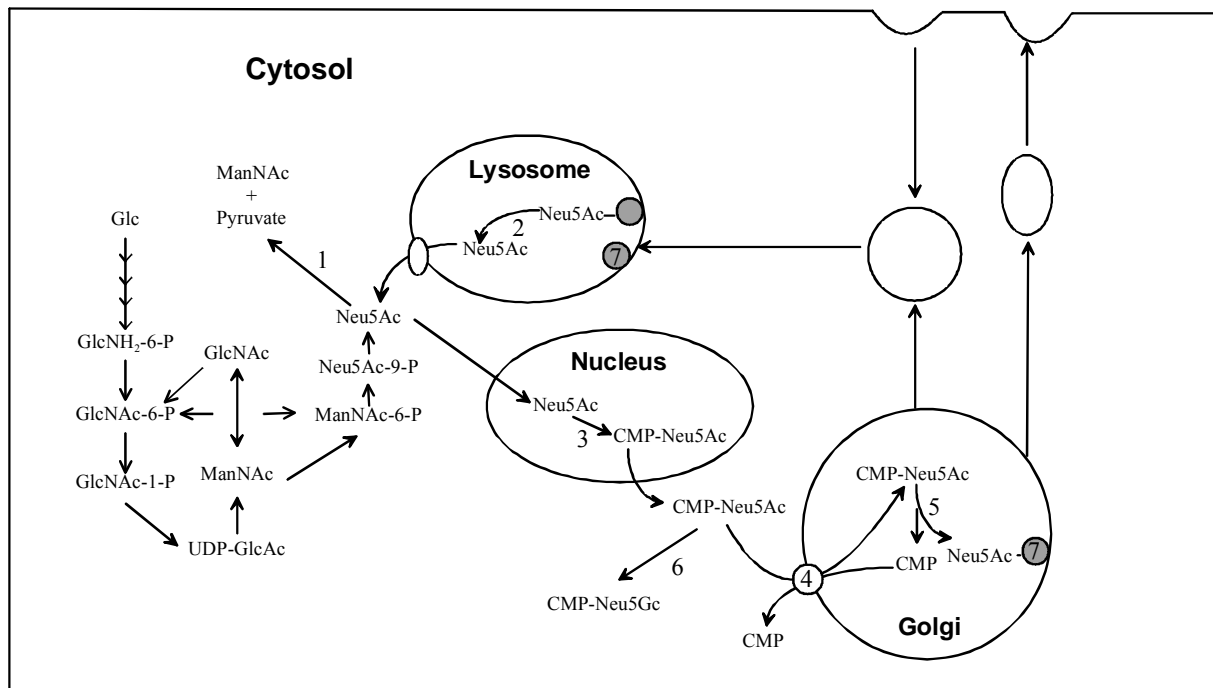


Figure 1-2 General Metabolic Pathway of Sialic Acids. The general pathway for the biosynthesis, activation, transfer, and eventual recycling of sialic acids is indicated. 1, sialate-pyruvate-lyase; 2, sialidase; 3, CMP sialic acid synthase; 4, CMP-sialic acid transporter; 5, sialyltransferase; 6, CMP-Neu5Ac hydroxylase; 7, glycoconjugates.

1.2.1 CMP-Sialic Acid Transporter

Once CMP-sialic acids are formed, they are transported into the lumen of the Golgi apparatus, where they are utilized for the sialylation of glycoconjugates [2]. The transport of CMP-sialic acid is mediated by a highly specific transporter [19-22]. In mammals, studies *in vitro* strongly suggest that the CMP-sialic acid transporter is an antiporter that delivers CMP-sialic acids into the Golgi lumen with the simultaneous counter-transport of CMP, the reaction product following sialylation of proteins and lipids [19;20]. The CMP-Neu5Ac transporter has a very high affinity for its substrate, with a K_M in the range of 1 to 10 μM [21-23]. Moreover, this procedure is not energy-dependent, and can be competitively inhibited by the corresponding nucleoside mono- and diphosphate, but not by sialic acids [19;22;24].

In the past few years, several CMP-sialic acid transporter genes have been cloned [24-28]. However, little is known about the regulation of CMP-sialic acid translocation, and the influence of CMP-sialic acid transporter on the sialylation.

1.2.2 Sialyltransferase

Sialyltransferases are a subset of the glycosyltransferase family that utilize CMP-sialic acids as the activated sugar donor to catalyze the transfer of sialic acid residues to terminal non-reducing positions of oligosaccharide chains on glycoproteins and glycolipids [2;29]. These enzymes have been shown to be restricted to the trans cisternae of the Golgi apparatus and the trans Golgi network [2]. However, in some cases, these enzymes may have a more diffuse distribution throughout the Golgi apparatus in some cells [30;31].

In general, each sialic acid linkage is generated by a single or more sialyltransferases which show the specificity not only for the linkage but also for the acceptor [2;29]. In mammalian systems, sialic acids are usually α 2,3- or α 2,6-linked to Gal and GalNAc of the glycan on glycoproteins. Further structural subdivision can be made according to the type of the underlying oligosaccharide chain involved, *i.e.*, *O*-linked or *N*-linked [2;29;32]. In gangliosides, sialic acids are found to be α 2,8-linked to other sialic acids [2]. Till now, a family of distinct sialyltransferase gene products have been identified that can transfer sialic acid onto the oligosaccharides on glycoproteins or gangliosides [29].

1.2.3 Metabolism of the *O*-Acetyl Group on Sialic Acids

The two main enzymes involved in the metabolism and catabolism of *O*-acetylated sialic acids are acetyl-coenzyme A:sialate-*O*-acetyltransferase (OAT) for the *O*-acetylation of sialic acids, and sialate-*O*-acetyltransferase (OAE) for the de-*O*-acetylation of *O*-acetylated sialic acids.

1.2.3.1 Acetyl-Coenzyme A:Sialate-O-Acetyltransferase (OAT)

At least two OATs are known to introduce acetyl group into sialic acids, using AcCoA as the donor substrate [33;34]. The acetyl-coenzyme A:sialate-4-*O*-acetyltransferase (EC 2.3.1.44; 4-OAT) [2;4] is responsible for the *O*-acetylation of the hydroxyl group located on the pyranose ring of sialic acid. The enzyme is absolutely specific with regard to the insertion site of the acetyl group at C-4. In contrast to 4-OAT, the exact primary attachment site of the acetyl-coenzyme A:sialate 7(9)-*O*-acetyltransferases (EC 2.3.1.45; 7(9)-OAT) [2;4] is not

known. What is known is that these enzymes incorporate acetyl groups onto the glycerol side chain of sialic acid, probably at position 7, followed by migration to C-9 [9;35]. Therefore, this enzyme is addressed as 7(9)-OAT. However, the available data do not exclude the possibility that two separate *O*-acetyl transferases specific for 7- and 9-*O*-acetylation exist.

Investigations on OATs in different tissues [5;6;35-38] show that these enzymes are membrane-bound, co-localized with sialyltransferases, and utilize glycosidically linked sialic acids as the acceptor substrate. *O*-Acetylation of sialic acids predominantly takes place in the Golgi apparatus, which requires the cooperation of a putative acetyl-CoA transporter [36]. Similar results were also obtained for the *O*-acetylation of gangliosides in Golgi-enriched vesicles from human melanoma cells [38].

However, a cytosolic 7(9)-OAT activity has been identified in bovine and equine submandibular glands, which could transfer acetyl groups from AcCoA to either exogenous or endogenous free sialic acids [39]. Till now, the existence of a true cytosolic enzyme is still an open question, which might result from the partial solubilization of membrane-bound enzyme during the homogenization procedure. However, the possibility that sialate *O*-acetylation happens prior to the sialylation of glycoconjugates cannot be excluded, since CMP-Neu5,9Ac₂ isolated from bovine submandibular glands has been found to be utilized by the STs in the microsomes of the same tissues [39;40].

Unfortunately, even though OATs were discovered some 30 years ago [33;34], they have proven intractable to be purified to homogeneity. Attempts to clone one of the sialate-*O*-acetyltransferase gene(s) have also been unsuccessful, and attempts at expression cloning resulted in the identification of proteins capable of inducing *O*-acetylation of sialic acids but not the enzyme itself [41;42]. Several groups have cloned genes encoding the putative AcCoA transporter [43-45], which can transfer AcCoA into the Golgi apparatus, and is involved in the *O*-acetylation of gangliosides. However, these putative AcCoA transporters show high degrees of homology to the putative transmembrane protein (EMBL) from *Saccharomyces cerevisiae* and *Caenorhabditis elegans*, in which neither gangliosides nor sialic acids are expressed. Ogura *et al.* (1996) [46] cloned a putative sialate-*O*-acetyltransferase specific for GD₃ but the deduced amino sequence showed significant homology with a mouse milk fat globule membrane protein. These data suggest that caution should be paid to the results obtained from expression cloning, because unrelated proteins can induce *O*-acetylation of sialic acids.

1.2.3.2 *Sialate-O-Acylesterase (OAE)*

O-Acetyl groups of sialic acids can be removed by esterases specific for *O*-acetylated sialic acids. Several of these enzymes have been found in viruses [47-51], bacteria [52] and vertebrates [53-55], showing different substrate specificities. The esterase specific for 4-*O*-acetyl groups of sialic acids is called sialate-4-*O*-acylesterase (4-OAE) [48;51], and the one for 9-*O*-acetyl groups is sialate-9-*O*-acylesterase (EC 3.1.1.53, 9-OAE) [53;54;56].

Influenza C virus specifically recognizes Neu5,9Ac₂ [57], and the receptor-destroying enzyme of this virus has been characterized as sialate-9-*O*-acylesterase [48]. This unique property of influenza C virus makes it a very useful and specific tool to investigate either free or glycosidically linked 9-*O*-acetylated sialic acids [58-60].

In mammals [53;55], most OAEs appear to be highly specific for the cleavage of 9-*O*-acetyl groups from sialic acids. However, esters from the 7- or 8-position can be indirectly removed [53], after they have migrated to the 9-position [9;35]. Moreover, an esterase specific for sialate-4-*O*-acetyl groups has been found in equine tissues [54].

The data currently available demonstrate the existence of two 9-OAEs belonging to the diisopropylfluorophosphate (DFP)-sensitive esterase family [61;62]. One is localized in the cytosol (Cse) and the other in the lysosomal/endosomal compartment (Lse). The latter is a water-soluble *N*-linked glycoprotein that transits the Golgi apparatus during its biosynthesis, and undergoes a late proteolytic cleavage possibly resulting in an increase in the enzyme activity [63]. Interestingly, a portion of the newly synthesized Lse is secreted as an uncleaved form [63], however, the biological significance of this enzyme form is unclear.

In contrast to Lse, Cse is a non-glycosylated protein [53;56;61]. Nevertheless, it has been reported that Lse and Cse of mouse liver could be encoded by one gene *via* differential usage of a signal peptide-encoding exon at the *N*-terminus [64]. This suggests that at least part of the Cse activity results from the alternate cytosolic form derived from the lse gene.

It has been postulated that Lse and Cse may play different roles in the catabolism of *O*-acetylated sialic acids [63;64]. Lse is believed to participate in the de-*O*-acetylation of 9-*O*-acetylated sialoglycoconjugates in the lysosomal compartment. However, it cannot access free *O*-acetylated sialic acids present in the cytosol, which are poor substrates for CMP-sialic acid synthase [65] and sialate-pyruvate-lyase [66]. Additionally, CMP-linked *O*-acetylated sialic acids are poor substrates for sialyltransferases [67]. Therefore, Cse has been proposed to act on the cytosolic pool of free 9-*O*-acetylated sialic acids. In this respect, it is interesting to note

that the Cse activity is very high in rat brain [53], where 9-*O*-acetylated sialic acids are commonly expressed.

1.2.3.3 Regulation of the Expression of *O*-Acetylated Sialic Acids

A great deal of evidence suggests that the expression of *O*-acetylated sialic acids is growth-, species-, tissue-, and molecule-specific [2], indicating that a complex combination of different factors is involved in regulating the expression of *O*-acetylated sialic acids on glycoconjugates. These factors might include the regulation of OATs expression, the control of substrate synthesis and transport, as well as the control of *O*-acetylated sialic acids degradation.

The expression of OAT gene might be regulated during cell differentiation [41;68], development [69] and in diseased cells [7]. However, the factors controlling the gene expression and the regulation of enzyme activities *in vivo* are still challenges for future research, since these enzymes have proven difficult to be purified or cloned [70].

Investigations on 7(9)-OATs point out the existence of a family of 7(9)-OATs having different substrate affinities [4]. It has been found that *O*-acetylation can be specific for particular types of glycoconjugates in a given cell type. For example, OAT from rat liver seems to be specific for Neu5Ac present on *N*-glycans [71], while that from human melanoma is specific for gangliosides [72]. Therefore, the activity of OAT could be regulated at the substrate level, that is, the availability of an appropriate substrate can influence the activity of OAT in a given cell type [41]. Actually, it was observed that the polyoma T antigen-positive Chinese hamster ovary cells (CHO-Tag cells), which do not express cell-surface 9-*O*-acetylation, could express *O*-acetylated sialic acids when transiently transfected with a cDNA encoding lactosamine-specific α 2,6-sialyltransferase (Gal β 4GlcNAc: α 2,6-sialyltransferase, ST6Gal I) [41].

The AcCoA present in the cytosol must be transported into the Golgi apparatus in order to act as the donor substrate for OATs [36;37]. A specific transport system has been proposed to regulate the supply of AcCoA [36]. Till now, the underlying mechanism of the transport of AcCoA has not been clarified.

The expression of *O*-acetylated sialic acids could further be regulated by OAEs. That is, the selective and specific distribution of OAEs could be another potential regulatory point. It has been demonstrated that the distribution of Lse mRNA is both spatially and temporally regulated during murine development [73]. In mouse adult tissues, the differential expression

of mRNAs for Lse and Cse was observed [63;64]. Additionally, large molecules such as cell surface mucins could play as a regulatory factor by masking some *O*-acetylated gangliosides [74], and thus hindering OAE activities.

1.2.4 Sialidase

Sialidases (EC 3.2.1.18) are classified into two groups according to their site of cleavage. Exo-sialidase hydrolyzes the *O*-glycosidic linkages between the terminal sialic acids and the subterminal monosaccharides of free and glycoconjugate-bound oligosaccharides, while endo-sialidase hydrolyzes the internal sialic acid glycosidic bonds of oligo- or polysialyl chains [2]. Most microbial and animal sialidases belong to the first group, and are usually simply referred to as “sialidase” [2].

1.2.5 Sialate-Pyruvate-Lyase

Sialic acids released by sialidases are degraded by a cytosolic sialate-pyruvate-lyase (EC 4.1.3.3), yielding acylmannosamines and pyruvate. Figure 1-3 shows the structural elements of sialic acids required for sialate-pyruvate-lyase recognition [66;75].

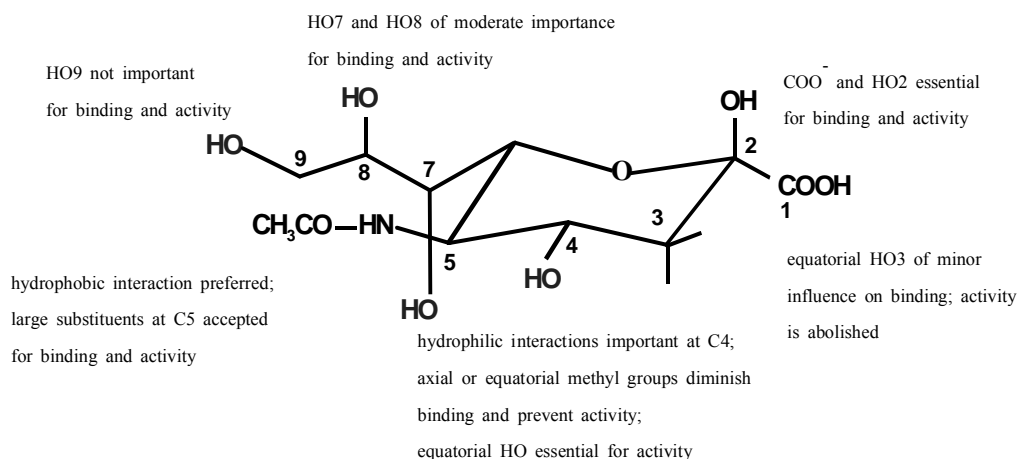


Figure 1-3 Sialic Acid Structural Features Required for Interaction with Sialate-Pyruvate-Lyase [75].

1.3 Methods for Studying Sialic Acids

For accurate analysis, sialic acids from biological sources must be released and purified without the loss of their modification. Practically, this is a severe problem when analyzing glycosidically linked *O*-acetylated sialic acids. Enzymatic hydrolysis using sialidase cannot completely release *O*-acetylated sialic acids, while 4-*O*-acetylated sialic acids are resistant to the action of sialidases with the exception of some viral sialidases [2]. In contrast, mild acid hydrolysis can release almost all kinds of sialic acids, however, a significant proportion of *O*-acetyl groups is lost [76].

Once released and purified, sialic acids can be analyzed by colorimetry, TLC (thin-layer chromatography), GLC (gas-liquid chromatography), MS (mass spectrometry) and NMR (nuclear magnetic resonance spectrometry) [2;8]. The technique of derivatization with DMB followed by HPLC analysis with fluorescent detection has proven to be particularly sensitive, specific, and applicable to most sialic acids [77]. Monoclonal antibodies and lectins have also been used to identify *O*-acetylated sialic acids [2;7;8]. A recombinant soluble form of the 9-*O*-acetyl-specific hemagglutinin of influenza C virus has been successfully used to probe for such molecules in cells and tissues [59;60].

1.4 Glycosylation Patterns of Human Colonic Mucins

1.4.1 Structure of Mucin-Type *O*-Glycan

Mucins are high molecular weight glycoproteins, which are heavily glycosylated with many oligosaccharide side chains, which are *O*-glycosidically linked to the protein backbone [78;79]. Three regions of complex oligosaccharides on mucin-type proteins have been arbitrarily assigned: the core region, which comprises the innermost two or three sugars of the glycan chain proximal to the peptide, the backbone region, which mainly contributes to the lengths of the chains formed by uniform elongation, and the peripheral region, which exhibits a high degree of structural complexity and can represent blood group and other related carbohydrate antigens [80]. Table 1-2 shows the eight core structures found in mucin-type *O*-glycan [78;80].

The pattern of *O*-linked glycans shows species-, tissue-, cell type-specificity, and could be changed in diseases [78;79;81]. The glycan structures of human colonic mucins are predominantly based on core 3 structures [82;83], and a multitude of structural alterations of *O*-glycans has been observed in colorectal cancer, including a decrease in total mucin content, a decrease of sulfatation and *O*-acetylation with an increase in sialylation [79;84-86].

Table 1-2 *O*-Glycan Core Structures.

Core	Structure
1	Gal β 1-3GalNAc α -Ser/Thr-R
2	GlcNAc β 1-6[Gal β 1-3]GalNAc α -Ser/Thr-R
3	GlcNAc β 1-3GalNAc α -Ser/Thr-R
4	GlcNAc β 1-6[GlcNAc β 1-3]GalNAc α -Ser/Thr-R
5	GalNAc α GalNAc α -Ser/Thr-R
6	GlcNAc β 1-6GalNAc α -Ser/Thr-R
7	GalNAc α 1-6GalNAc α -Ser/Thr-R
8	Gal α 1-3GalNAc α -Ser/Thr-R

1.4.2 *O*-Acetylated Sialic Acids on Human Colonic Mucins and Human Colorectal Cancer

The mucins synthesized and secreted by the human colonic mucosal cells constitute the major component of mucus gels that cover the epithelial surfaces of colon [87], and thus protect cells and epithelia from the attack by the external environment, including degradative enzymes [88], bacteria [89] and mechanical stresses [90]. Membrane-bound mucins may have protective functions comparable to secretory mucins, however, they may also play a role in mediating cell-cell interactions especially during development and carcinogenesis, as well as in cell migration, adhesion and recognition [90;91].

The majority of oligosaccharides in normal colonic mucin contain 4-12 monosaccharides, and are highly sialylated and sulfated [82;83;92]. Chemical analyses have shown that more than 50 % of colonic mucin sialic acids are *O*-acetylated, with 30 % containing oligo-*O*-acetyl forms with 2 or 3 acetyl esters per sialic acid residue [93-95]. Histochemical data suggest that the total sialate *O*-acetylation is at least 80 % [96]. This high degree of sialate *O*-acetylation is characteristic for the human colon and has not been detected in other human tissues [95],

although a high level of *O*-acetylated sialic acids has been observed in bovine submandibular gland mucins [18].

The occurrence of *O*-acetylated sialic acids in human colonic tissue is developmentally regulated, with the level of sialate *O*-acetylation strongly increasing after birth [69]. However, this level can be quite variable, depending on bacterial products and environmental stimuli [69]. Moreover, an ethnic difference in the expression level of *O*-acetylated sialic acids on colonic mucins has been observed between Sino-Japanese and non-Sino-Japanese [97]. The occurrence of *O*-acetylated sialic acids in the latter group (British, Icelanders, South African blacks and Bahrainis) is much more frequent than in Chinese and Japanese. This is speculated to be due to a single autosomal gene polymorphism, which might be related to a different infective environment.

The significance of the high expression level of *O*-acetylated sialic acids is related to the degradation of mucins by bacterial enzymes [52]. The presence of ester groups on sialic acids can hinder the action of enteric bacterial sialidase [98;99]. However, a sialate-*O*-acetyl esterase has been detected in individual bacterial strains and in faecal extracts from normal individuals [52;100], which can facilitate the action of sialidase by removing *O*-acetyl groups from sialic acids. The lower activity of the esterase relative to sialidase suggests that the degradation of mucins could be regulated at the level of sialate *O*-acetylation [100].

In contrast to melanoma and basalioma [17;101;102], the degree of *O*-acetylation, presumably on *O*-linked oligosaccharides, in cancerous colonic mucosa appears to be reduced in comparison to that normally found in healthy colonic mucosa [93-95;103]. Further studies on the carcinoma human colonic cell lines by Corfield *et al.* (1999) [95] show that di- and tri-*O*-acetylated sialic acids are diminished at the early stage of malignant transformation, while a significant proportion of mono-*O*-acetyl sialic acid remains constant in the range of 10-15 % of membrane sialic acids. These observations raise many questions concerning the occurrence and enzymatic processes involved into the *O*-acetylation and de-*O*-acetylation of sialic acids in human colon.

1.5 Objective of this Project

The majority of our knowledge concerning the *O*-acetylation of sialic acids in mammals has been obtained from the investigations using bovine submandibular glands and rat liver, however, these enzymes have not been purified to homogeneity or cloned. This requires

further investigation to obtain much more knowledge about the sialate *O*-acetylation in different systems.

The high level of *O*-acetylated sialic acids in human colonic tissues makes this tissue to be a good material for the investigation of enzymatic sialate *O*-acetylation, which is not yet well understood. Therefore, one of the main aims of this project is to demonstrate the enzyme activity in human colonic mucosa, find the enzyme acceptor substrate, confirm the nature of enzyme products, and determine the kinetic parameters. On this basis, attempts would be made to solubilize the membrane enzyme in an active form and, if successful, further purification of the enzyme would be performed. Another main point of this project is to work out the pathway of enzymatic sialate *O*-acetylation in human colonic mucosa.

Moreover, the reduction of *O*-acetylated sialic acids has been observed to be an early event during the malignant transformation in human colorectal cancer, however, the underlying mechanism is not clear. It is supposed that changing of the activities of OAT and OAE could occur in cancerous tissues, thus, the comparative investigation of the activities of these two enzymes in the uninvolved and cancerous colonic mucosa will be performed. This investigation can shed light on the regulation of *O*-acetylation of sialic acids at the biochemical level in human colonic mucosa as well as on the possible mechanism for the decrease in the *O*-acetylated sialic acids level in cancerous tissues.

2 Materials and Methods

2.1 Materials

2.1.2 Chemicals and Enzymes

Chemicals	Company
[³ H]AcCoA	Moravek Biochemicals, Ca. USA
AcCoA, trilithiumsolt	Sigma, Deisenhofen
Acetic acid, 100 %	Merck, Darmstadt
Acetic anhydride	Riedel de Haßn
Acetic acid kit	Boehringer, Mannheim
Asialofetuin	Sigma, Deisenhofen
4-(2-amionethyl)-benzolsulfonylchloride- hydrochloride (Pefabloc)	Boehringer, Mannheim
Ammonia, 25 %	Merck, Darmstadt
Ammonium acetate	Merck, Darmstadt
BCA protein kit	Pierce, Illinois, USA
n-Butanol	Riedel de Haßn
Bovine serum albumin	Boehringer, Mannheim
Cacodylate sodium	Merck, Darmstadt
Calcium chloride	Fluka, Buchs, Schweiz
CHAPS	Biomol, Hamburg
<i>p</i> -Chloromercuribenzoate	Sigma, Deisenhofen

Chemicals	Company
Chloroform	Merck, Darmstadt
Cytidine 5'-monophosphate, disodium salt	Sigma, Deisenhofen
CMP-[4,5,6,7,8,9- ¹⁴ C]Neu5Ac	Amersham, Braunschweig, Germany
CMP-Neu5Ac	Calbiochem-Novabiochem, Bad Soden
CoA, trilithiumsalt	ICN, Aurora, Ohio, USA
Complete TM	Roche, Mannheim, Germany
Diethylpyrocarbonate	Applichem, Darmstadt
4,4'-Diisothiocyanostilbene-2,2'-disulfonic acid disodium salt (DIDS)	Sigma, Deisenhofen
Dipotassium phosphate	Merck, Darmstadt
Dithiothreitol	Biomol, Hamburg
DMB	Dojindo Laboratories, Tokyo, Japan or Fluka, Buchs, Schweiz
Edible bird nest substance	Mect. Co., Tokyo, Japan
Ethylenediaminetetraacetic acid, tetrasodium salt	Sigma, Deisenhofen
Ethanol	Merck, Darmstadt
Formic acid	Merck, Darmstadt
Fetuin	ICN, Aurora, Ohio, USA
Galactose-MU	Sigma, Deisenhofen

Chemicals	Company
5-Fluoro-2',3'-isopropylidene-5'- <i>O</i> -(4- <i>N</i> -cetyl-2,4-dideoxy-3,6,7,8-tetra- <i>O</i> -acetyl-1-methoxycarbonyl-D-glycero- α -D-galactooctapyranosyl)uridine (KI-8110)	Mect Corporation, Tokyo, Japan
Glycerol	Merck, Darmstadt
GD ₃	Biomol, Hamburg
Human colonic mucin	Kindly presented by Prof. Dr. K. Ishihara, Kitasato University, Sagamihara, Japan
Lecithin	Sigma, Deisenhofen
LUBROL Px	Sigma, Deisenhofen
Methanol	Merck, Darmstadt
MgCl ₂	Merck, Darmstadt
Monopotassium phosphate	Merck, Darmstadt
Monoclonal antibody for 9- <i>O</i> -acetylated GD ₃	Kindly presented by Mr. S. Toshiaki, Snow Brand Milk Products Co. Ltd., Kawagoe, Japan
Methylumbelliferon	Sigma, Deisenhofen
4-Methylumbelliferyl acetate	Sigma, Deisenhofen
Nitroblautetrazoliumchlorid /5-Brom-4-chlor-3-indolyl-phosphate (NBT/BCIP stock solution)	Boehringer, Mannheim
Neu2en5Ac	Boehringer, Mannheim

Chemicals	Company
Neu5Ac	Snow Brand Milk Products Co. Ltd., Kawagoe, Japan
Nonidet P 40	Fluka, Buchs, Schewiz
9- <i>O</i> -Acetylated GD ₃	Prof. Bernhard Kniep, Institut für Immunologie, Medizinische Fakultät, "Carl Gustav Carus" Universität, Dresden
1- <i>O</i> -Octyl-β-D-glucopyranoside	Fluka, Buchs, Schewiz
Orcinol	Fluka, Buchs, Schewiz
Perchloroacetic acid, 37 %	Merck, Darmstadt
Pefabloc SC	Roche, Germany.
n-Propanol	BASF, Ludwigshafen
Potassium chloride	Merck, Darmstadt
Propionic acid, 99 %	Merck, Darmstadt
Pyridine	Merck, Darmstadt
Saponin	Sigma, Deisenhofen
α2,6-Sialyltransferase, rat liver	Sigma, Deisenhofen
Sialate-pyruvate-lyase	Sigma, Deisenhofen
Sodium hydroxide	Merck, Darmstadt
Sucrose	Carl Roth GmbH, Karlsruhe
Tris (hydroxymethyl)aminomethane, ultra pure	ICN, Aurora, Ohio, USA

Chemicals	Company
Triton X-100	Sigma, Deisenhofen
Trichloroacetic acid	Merck, Darmstadt
Trypsin	Sigma, Deisenhofen
Tween 20	Sigma, Deisenhofen
Zwittergent 3-12	Fluka, Buchs, Schewiz

2.1.2 Materials for Chromatography

Chemicals	Company
Methanol for HPLC	Promochem, Wesel
Acetonitrile for HPLC	Promochem, Wesel
Dowex 2 x 8, 200-400 mesh	Fluka, Buchs, Schweiz
Dowex 50W x 8, 20-50 mesh	Fluka, Buchs, Schweiz
DC-Cellulose plate, 20 x 20 cm	Merck, Darmstadt
DEAE-Sepharose FF	Pharmacia, Freiburg
HPTLC silica gel 60 plate, 10 x 20 cm	Merck, Darmstadt
RP-18 cartridge column	Merck, Darmstadt

2.1.3 Biological Materials

Human colonic tissues were prepared freshly from patients who underwent surgical resection of colorectal carcinoma. Each patient was informed about the study and gave written consent in accordance with the ethical guidelines of the Christian-Albrechts-University (CAU), Kiel.

Fresh “uninvolved” colonic mucosa was obtained from the excised end of colonic tissue resected for carcinoma, and was found to be histologically normal. For the comparative study,

the cancerous mucosa, containing at least 80 % cancer cells, was also prepared from the same patient. The cancer stage assessment, based on the TNM classification system [104], was made by routine clinical and histological methods. Of the 12 tumor samples obtained, 5 were at stage 2, 5 were at stage 3 and 2 were at stage 4. The dissected mucosa was washed in PBS and frozen at -80 °C until required.

Two non-malignant human colonic epithelium cell lines (csc-das1 and ncm-das 460) were kindly provided by Professor Dr. H. Kalthoff (Klinik für Allgemeine Chirurgie und Thoraxchirurgie, Christian-Albrechts-Universität zu Kiel, Germany)

2.1.4 Instruments and Other Materials

Instruments and Materials	Company
Centrex UF-0.5 and UF-2	Schleicher & Schuell, Dassel
Dialysis Tube	Sartorius AG, Göttingen
Eppendorf Thermostat 5320	Eppendorf, Hamburg
Fluorolite	Dynatech Lab., Inc. Chantilly, VA
HPLC System	Merck-Hitachi, Darmstadt
J2-HS Centrifuge	Beckman, München
Lyophilizer alpha 1-4	Bachofer, Reutlingen
Microplate	Sarstedt, Nümbrecht
Microplate Photometer, Titertek Multiscan-MC	Flow Laboratories, Uxbridge, U.K.
Mini-Potter Homogenizer	Walter Ebel, Hamburg
Quartz Cuvette, 1 mL	Hellma, Müllheim
U-2000 Spectrophotometer	Hitachi, Japan

Instruments and Materials	Company
Speed Vacuum, SC 110	Bachofer, Reutlingen
Sunrise, Microplate Photometer	TECAN, Crailsheim, Germany
TLC-Linear Analyzer LB 284	Berthold, Wildbad, Germany
Ultracentrifuge L7-65	Beckman, München
Ultraturrax IKA T25	Jahnke & Kunkel, Stufen
Ultrasonic Processor	Walter F.C. EBEL, Hamburg, Germany

2.2 Methods

2.2.1 Preparation of Subcellular Fractions from Human Colonic Mucosa

2.2.1.1 *Preparation of Microsomes from Human Colonic Mucosa*

All procedures were carried out at 4 °C unless otherwise stated. The mucosa was homogenized in 10 mM Tris-HCl buffer, pH 7.4, using an Ultraturrax at maximal speed (3 times 10 s with a 30 s pause on ice between each burst) and centrifuged at 10 000 x g for 15 min. The supernatant (crude homogenate) was centrifuged at 100 000 x g for 60 min. The resulting supernatant (cytosolic fraction) was stored at -80 °C until required. The resulting pellet was re-homogenized with a mini Potter homogenizer in the same buffer and centrifuged again at 100 000 x g for 60 min. The pellet was re-suspended in PK buffer (50 mM phosphate buffer, pH 7.0), containing 50 mM KCl, and referred to as the microsomal fraction.

2.2.1.2 *Simultaneous Preparation of Microsomes and Lysosomes for Sialate-O-Acetyltransferase Assay*

Lysosomes and microsomes were prepared from human colonic mucosa using the method described in Butor *et al.* (1993) [71]. Briefly, the mucosa was homogenized in 10 mM Tris-HCl buffer, pH 7.45, containing 0.25 M sucrose as described above. Following centrifugation at 3 000 x g for 15 min, the supernatant (referred to as homogenate) was removed and further centrifuged at 11 000 x g for 30 min. The pellet (post 11 000 x g pellet) was saved for the

preparation of lysosomes, while the resulting supernatant was centrifuged at 100 000 x g for 60 min. The pellet and supernatant obtained were referred to as microsome and cytosol, respectively.

The post 11 000 x g pellet was re-suspended in 0.025 M sucrose (1 mL/g of mucosa) and allowed to swell for 30 min on ice. The supernatant obtained after centrifugation at 11 000 x g for 20 min was removed and set aside, while the pellet was re-suspended in 0.025 M sucrose containing 0.02 M KCl (0.5 mL/g of mucosa) and again centrifuged at 11 000 x g for 30 min. The supernatants were pooled (crude lysosomal fraction) and used for esterase assay.

A total membrane fraction was also prepared from the homogenate through centrifugation at 100 000 x g for 30 min. The resulting pellet, which contained all membrane components, was surfaced washed, re-suspended in PK buffer containing 50 mM KCl, and stored at -80 °C until required.

2.2.1.3 *Preparation of Golgi Vesicles from Human Colonic Mucosa*

Procedure 1: Subcellular membrane fractions were isolated from human colonic mucosa using a modification of the discontinuous sucrose density gradient method described by Lepers *et al.* (1989) [21]. Briefly, 10 g of human colonic mucosa was homogenized in 30 mL of ice-cold homogenizing buffer (10 mM Tris-HCl buffer, containing 5 mM MgCl₂, pH 7.4) containing 0.25 M sucrose, using an Ultraturrax at intermediate speed (3 times 10 s with 30 s pause on ice between each burst). Following centrifugation at 600 x g for 15 min, the resulting supernatant was adjusted to 1.4 M sucrose by the addition of 2 M sucrose. All sucrose solutions were prepared in the homogenizing buffer. Ten mL of the 1.4 M sucrose supernatant fraction was layered over a discontinuous sucrose gradient consisting of 5 mL of 1.6 M sucrose, 9 mL of 1.2 M sucrose and 9 mL of 0.8 M sucrose in a Beckman SW 28 centrifuge tube. After centrifugation at 100 000 x g for 2 h, the turbid bands at different sucrose interfaces were collected, diluted to 0.25 M sucrose with homogenizing buffer and collected by centrifugation at 100 000 x g for 30 min. The resulting pellet was re-suspended in PK buffer containing 50 mM KCl and 0.25 M sucrose, aliquoted and stored at -80 °C until required. The fraction collected from the 0.8 M/1.2 M interfaces represented the Golgi-enriched fraction (fraction A). Fractions collected from the 1.2 M/1.4 M and the 1.4 M/1.6 M interfaces, as well as the pellet, were referred to as fractions B, C and D, respectively.

Procedure 2: Golgi vesicles were prepared by a modification of the method described in Sjoberg *et al.* (1993) [38]. All sucrose solutions were prepared in 25 mM of phosphate buffer, pH 7.0. Human colonic mucosa was homogenized in 3 volumes of 0.25 M sucrose using an Ultraturrax as described above, and centrifuged at 500 x g for 15 min. The supernatant was adjusted to 1.2 M sucrose using a 2 M sucrose solution, of which 18 mL was placed in a 35 mL ultracentrifuge tube. The 1.2 M suspension was overlaid with 10 mL of 1.1 M sucrose followed by 7 mL of 0.25 M sucrose. The membrane fraction removed from the 0.25-1.1 M interface following centrifugation at 85 000 x g for 90 min was adjusted to 0.25 M sucrose and pelleted at 100 000 x g for 30 min. The pellet was resuspended in PK buffer containing 50 mM KCl and 0.25 M sucrose, aliquoted, and stored at – 80 °C until required.

Procedure 3: Subcellular membrane fractions were isolated from human colonic mucosa using the discontinuous Ficoll density gradient method described by Vandamme-Feldhaus and Schauer (1998) [35]. Human colonic mucosa was homogenized in 1.5 volumes of 100 mM Tris-HCl buffer, pH 7.4, using an Ultraturrax and adjusted to 1.5 % Ficoll by the addition of 3 % Ficoll solution containing protease inhibitors. All the Ficoll solutions were prepared in 100 mM Tris-HCl buffer, pH 7.4. After centrifugation at 600 x g for 15 min, the supernatant was laid over a discontinuous Ficoll gradient consisting of 10.8 mL of 19 % Ficoll, and 5.4 mL each of 17 %, 15 %, 9 % Ficoll in a SW 28 ultracentrifugation tube. Following centrifugation at 100 000 x g for 30 min, the membrane fractions at different Ficoll interfaces were collected, diluted 10 times with 10 mM Tris-HCl buffer, pH 7.4, and pelleted (100 000 x g for 30 min). The resulting pellet was re-suspended in PK buffer containing 50 mM KCl and 0.25 M sucrose, aliquoted, and stored at – 80 °C until required.

Procedure 4: The sucrose-CsCl density gradient method described by Sandberg *et al.* (1980) [105] and Yusuf *et al.* (1983) [106] was adapted for the preparation of Golgi vesicles from human colonic mucosa. All sucrose-CsCl solutions were in PK buffer containing 50 mM KCl. Briefly, human colonic mucosa was homogenized in 3 volumes of ice-cold 0.3 M sucrose using an Ultraturrax and centrifuged at 10 000 x g for 15 min. The post-nuclear supernatant was mixed with 2 M sucrose and 1 M CsCl to a final concentration of 1.05 M and 10 mM, respectively. The sucrose-CsCl gradient was prepared by layering 5 mL of 1.0 M sucrose and 7 mL of 0.3 M sucrose over 25 mL of the 1.05 M sucrose/10 mM CsCl suspension. After centrifugation at 100 000 x g for 3 h, the Golgi-enriched fraction was collected from the 0.3-1.0 M interface, diluted with 10 mM Tris-HCl buffer and pelleted

(100 000 x g for 30 min). Pellets obtained were re-suspended in PK buffer containing 50 mM KCl and 0.25 M sucrose, aliquoted, and stored at – 80 °C until needed.

2.2.1.4 *Preparation of the Cytosolic and Membrane Fractions from Human Colonic Cell Line*

The method described in Vavasseur *et al.* (1994) [107] was adopted to prepare the cytosolic and membrane fractions. In short, the cell pellets were treated with sonication (Power setting 20, 3 times 15 second) in 250 µL of PK buffer containing 50 mM KCl. After centrifugation at 600 x g for 15min, the resulting supernatant (referred as homogenate) was further centrifuged at 100 000 x g for 30min. The resulting supernatant (cytosolic fraction) and the pellet (membrane fraction) as well as the homogenate were immediately used for 7(9)-OAT assay.

2.2.2 Protein Determination

Protein concentration was measured using the Micro-BCA protein assay reagent kit as described by the manufacturer.

2.2.3 Marker Enzyme Assay

2.2.3.1 *β-Galactosidase*

The lysosomal marker, β-galactosidase, was assayed by an adaptation of the method described in Lo *et al.* (1979) [108]. The reaction mixture, consisting of 50 mM sodium acetate (pH 4.6), 0.556 mM MU-Gal and varying amounts of protein in a final volume of 100 µL, was incubated at 37 °C for 30 min. The reaction was stopped by the addition of 600 µL of 0.5 M glycine (pH 10.2) and the fluorescence measured at excitation 365 nm and emission 400 nm on a Fluorescence Spectrophotometer (Dynatech Laboratories Inc.). The amount of MU released was determined from a MU standard curve (0-10 nmol). One unit of enzyme activity equals 1 nmol of MU released from MU-Gal per minute under the condition used.

2.2.3.2 *Sialyltransferase Assay Using Asialofetuin as Acceptor Substrate*

Sialyltransferase (ST) activity, a marker for the Golgi apparatus, was determined using the method described by Alhadeff and Holzinger (1979) [84] with slight modifications. In brief, the reaction mixture containing, in a final volume of 200 µL, 50 µg of membrane protein, 50 mM Tris-HCl buffer, pH 7.4, 10 mM MgCl₂, 200 µg asialofetuin, 1 % Triton X-100, and

CMP-[¹⁴C]Neu5Ac (0.025-0.01 μ Ci, 5 nmol) was incubated at 37° C for between 30 to 60 min. The reaction was terminated by the addition of 800 μ L ice-cold 5 % PCA to a final concentration of 4 %. The precipitates, following a 30 min incubation on ice, were collected by centrifugation at 14 000 rpm for 30 min. The resulting supernatant was removed, and the pellet was re-suspended in 200 μ L of ice-cold water with brief sonication. Following re-centrifugation at 14 000 rpm for 30 min, the pellet was dissolved in 400 μ L of 1 M NaOH by heating at 80 °C for 15 min. The dissolved material was then neutralized with 200 μ L of 2 M HCl and 550 μ L of this mixture was used to determine the incorporated radioactivity by scintillation counting. One unit of ST activity is defined as the amount of enzyme that will transfer 1 nmol of Neu5Ac from CMP-Neu5Ac to asialofetuin per hour under the condition used.

The latency of ST activity against asialofetuin was determined in the presence and absence of 0.5 % (v/v) of Triton X-100.

2.2.4 Sialic Acid Analysis

2.2.4.1 Acid Release of Glycoconjugate-bound Sialic Acids

Glycoconjugate-bound sialic acids were released with 2 M propionic acid at 80 °C for 2.5-4 h [76]. Following acid hydrolysis, sialic acids were collected by ultrafiltration (Centrex UF-0.5, 3 K MWCO) and purified as described below.

Free sialic acids were directly collected by ultrafiltration (Centrex UF-0.5, 3 K MWCO), lyophilized and purified.

2.2.4.2 Sialic Acid Purification

For the purpose of GC-MS analysis, sialic acids were purified by sequential ion exchange chromatography as described by Reuter and Schauer (1994) [109]. Acid-released sialic acids were applied to a column of the cation exchanger Dowex 50 W X 8 (20-50 mesh, H⁺ form) and eluted with 5-10 column volumes of water. The eluate was then applied to a column of the anion exchanger Dowex 2 X 8 (200-400 mesh, HCOO⁻ form). After washing the column with 5-10 column volumes of ice-cold water, sialic acids were eluted with 10 column volumes of 0.8 M HCOOH. The eluate was lyophilized and stored at -20 °C until analyzed.

For other purposes, sialic acids were purified on a column of Dowex 2 X 8 as described above.

2.2.4.3 *Quantitation of Sialic Acids*

Quantitation of sialic acids was carried out by a micro-adaptation of the Oricinol/Fe³⁺/HCl assay (BIAL-test) described in Reuter and Schauer (1994) [109]. Briefly, 100 µL of sample solution, containing up to 8 µg/µL sialic acids, was thoroughly mixed with 100 µL of BIAL reagent, and incubated at 95 °C for 15 min. After cooling on ice for 5 min, 700 µL of isoamylalcohol was added and the mixture was thoroughly vortexed. The absorbance of the upper organic phase, following centrifugation at 14 000 rpm for 15 min, was measured spectrophotometrically at 572 nm.

2.2.4.4 *Saponification of O-Acetylated Sialic Acids*

Sialic acids were incubated with 100 µL of 100 mM NaOH at 37 °C for 30 min, and the mixture was neutralized with an equal volume of 100 mM HCl. Alternatively, sialic acid samples were mixed with 5 volumes of 4 M NH₃ and incubated for 4 h at room temperature. The NH₃ was removed by lyophilization using a Speed Vacuum.

2.2.4.5 *Sialate-9-O-Acetylerase Treatment*

Fifteen µL of purified sialic acids was mixed with 25 µL of PBS (pH 7.0) and incubated with 15 µL of influenza C/JJ/50 virus at 37 °C for 1 h. The reaction was stopped by heating at 95 °C for 3 min, following which the suspension was cooled on ice for 5 min. After centrifugation at 14 000 rpm for 30 min, the supernatant was removed and analyzed by scintillation counting, as well as radio-HPLC.

2.2.4.6 *Sialate-Pyruvate-Lyase Treatment*

Purified sialic acids were incubated with 25 mU of sialate-pyruvate-lyase in 100 µL of PK buffer at 37 °C for 2 hour. The reaction was terminated by heating at 95 °C for 5 min and cooled on ice for 5 min. After centrifugation at 14 000 rpm for 30 min, the supernatant was removed and analyzed by scintillation counting, as well as radio-HPLC.

2.2.4.7 *Fluorometric HPLC Analysis of Sialic Acids*

Purified sialic acids were derivatized with DMB reagent (56 °C for 1 h, in the dark) and analyzed by fluorometric HPLC using the method described by Hara *et al.* (1989) [77]. A RP-18 cartridge column was eluted in the isocratic mode with methanol-acetonitrile-water

(7:9:84, by volume) at 1 mL/min. The retention times of the various sialic acids detected by HPLC were compared with authentic sialic acid standards prepared from bovine submandibular gland mucins.

2.2.4.8 *Preparation of Sialic Acids from Human Colonic Cell Lines*

Two human colonic cell lines were grown in RPMI medium supplemented with 10 % FCS containing 2 g/L NaHCO₃. Cells were harvested by centrifugation when confluent, washed twice with PBS, and detached with a cell scraper. The cell pellets were re-suspended in 2 M propionic acid and incubated at 80 °C for 4 h. Following extensive dialysis against distilled water (overnight at 4 °C), the released sialic acids were purified and used for fluorometric HPLC analysis as described above.

2.2.4.9 *Gas Chromatography-Mass Spectrometry*

Purified sialic acids were converted to their pertrimethylsilylated ester/ether derivatives and applied to a GC-system coupled with EI-MS and analyzed according to a fragmentation scheme by Kamerling *et al.* (1975) [110]. Professor Dr. J.P. Kamerling and Dr. G. J. Gerwig from The Bijvoet Center for Biomolecular Research, Department of Bio-Organic Chemistry, Utrecht, and The Netherlands, kindly performed these analyses.

2.2.5 *Acetyl-CoA: Sialate-7(9)-O-Acetyltransferase (7(9)-OAT) Assay*

2.2.5.1 *Assay for Membrane-Bound 7(9)-OAT*

Assay 1: Utilizing Endogenous Substrates, or Edible Bird Nest Substance (EBNS) as well as De-O-Acetylated Human Colonic Mucins (HCM) as Exogenous Acceptor Substrate

The 7(9)-OAT assay based on endogenous sialic acids present in the protein fractions from human colonic mucosa as the acceptor substrate was carried out in 30 µL of PK buffer containing 50 mM KCl, protease and esterase inhibitors (CompleteTM and Pefabloc, respectively), [³H]AcCoA (0.2 µCi, 8.3 µM) and 50 µg protein, usually at 37 °C for 15 min. The reaction was stopped with 60 µL of 3 M propionic acid to yield a final propionic acid concentration of 2 M. Glycoconjugate-bound sialic acids were released by incubating at 80 °C for 2.5 hours. Following the removal of proteins by centrifugation at 14 000 rpm for 30 min, the supernatant was lyophilized. The resulting residue was re-suspended in 200-300 µL of ice-cold water and applied to a Dowex 2 X 8 column (0.5 mL bed volume, 200-400 mesh,

HCOO⁻ form). After washing the column with 5 mL of water, sialic acids were eluted with 10 mL of 0.8 M formic acid. The eluate was lyophilized and taken up in 15 μ L water. One third of this sample was used to determine the total incorporated radioactivity by scintillation counting. The remaining portion was analyzed by radio-TLC. Following analysis using an automatic TLC-linear analyzer LB 284 (Berthold, Wildbad, Germany), the enzyme activity was determined by integrating the resulting radioactive peak and correlating this with the protein concentration. One unit is defined as the amount of enzyme that will transfer 1.0 pmol of acetate from AcCoA to sialic acid per minute at 37 °C, pH 7.0.

Enzyme assay utilizing edible bird nest substance (EBNS), containing 96 μ g sialic acids, or de-*O*-acetylated human colonic mucin (HCM) as exogenous acceptor substrate was carried out as described above.

Assay 2: Utilizing Neu5Ac or CMP-Neu5Ac as Exogenous Acceptor Substrate

The enzyme assay was carried out in 30 μ L of PK buffer containing 50 mM KCl, 600 μ M Neu5Ac or CMP-Neu5Ac, 50 mM KCl, protease and esterase inhibitors (CompleteTM and Pefabloc, respectively), [³H]AcCoA (0.2 μ Ci, 8.3 μ M) and 50 μ g microsomal protein, usually at 37 °C for 15 min. The reaction was stopped with 60 μ L of 3 M propionic acid and heated at 80 °C for 15 min. After the removal of proteins by centrifugation at 14 000 rpm for 30 min, the supernatant was lyophilized. The isolated sialic acids, following purification, were analyzed by radio-TLC.

Assay 3: Utilizing α 2,3- or α 2,6-Sialyllactose as Exogenous Acceptor Substrate

The enzyme assay utilizing 600 μ M of α 2,3- or α 2,6-sialyllactose, isolated from bovine colostrum, as the potential acceptor substrate was performed essentially as described above except that the assay was stopped by incubating at 95 °C for 5 min. After centrifugation at 14 000 rpm, the supernatant was lyophilized and analyzed by radio-TLC.

Assay 4: Utilizing GD₃ as Exogenous Acceptor Substrate

The enzyme assay was performed in 30 μ L of PK buffer containing 600 μ M GD₃, protease and esterase inhibitors (CompleteTM and Pefabloc, respectively), [³H]AcCoA (0.2 μ Ci, 8.3 μ M) and 50 μ g microsomal protein, usually at 37 °C for 15 min. The reaction was terminated with 120 μ L of methanol:chloroform (2:1, by volume). After the removal of proteins by centrifugation at 100 000 x g for 15 min, the supernatant was evaporated, re-suspended in 15 μ L of methanol:chloroform (2:1, by volume) and analyzed by radio-TLC.

2.2.5.2 *Assay for Solubilized 7(9)-OAT*

Solubilized proteins from microsomes were incubated with [³H]AcCoA (0.2 μCi, 8.3 μM) and 600 μM CMP-Neu5Ac in 30 μL of PK buffer containing protease and esterase inhibitors (CompleteTM and Pefabloc, respectively). The reaction was initiated with the addition of 0.8 μL of [³H]AcCoA and incubated at 37 °C for 15 min. After chasing with 0.8 μL of AcCoA for another 15 min, the reaction was stopped either with 60 μL of 3 M propionic acid to yield a final propionic acid concentration of 2 M, or with ice-cold ethanol (70 % final concentration) and incubated on ice for 30 min. The enzyme products were purified as described earlier (section 2.2.5.1) and analyzed by radio-TLC.

2.2.6 **Confirmation of the Nature of Enzyme Products**

2.2.6.1 *Radio-Thin-Layer Chromatography (Radio-TLC)*

The radiolabeled sialic acids released with propionic acid following 7(9)-OAT assays using endogenous or exogenous acceptor substrates were applied to TLC plates, either silica gel 60 HPTLC plates (10 x 10 cm) or cellulose plates (20 x 20 cm), together with authentic Neu5,9Ac₂ isolated from bovine submandibular glands. Silica gel plates were developed in methanol:chloroform:20 mM CaCl₂ (5:4:1, by volume) and cellulose plates were developed in propanol:butanol:0.1 M HCl (2:1:1, by volume) [109] or EtOH:1 M NH₄OAc (7:3, by volume, pH 7.3) [111].

Radiolabeled sialyllactose was applied to silica gel 60 HPTLC plates (10 x 10 cm) and developed in ethanol:pyridine:water:acetic acid:n-butanol (100:10:10:3:3, by volume) [112].

Radiolabeled GD₃, together with authentic 9-*O*-acetylated GD₃, was applied to silica gel HPTLC plates which were developed in methanol:chloroform:20 mM CaCl₂ (4:5:1, by volume) [109].

All radiolabeled sialic acids were detected using an automatic TLC-linear analyzer, and their migration distances were compared to those of sialic acid standards stained with Fe³⁺/orcinol/HCl (BIAL reagent).

Saponification of *O*-acetylated sialic acids was performed by incubating TLC plates overnight at room temperature in a tightly sealed chamber saturated with ammonium vapor. Plates treated in this manner were thoroughly dried under a stream of cold air and re-analyzed by TLC-linear analyzer.

2.2.6.2 *HPLC Analysis of Radiolabeled O-Acetylated Sialic Acids*

Following the incubation of microsomal fractions with CMP-Neu5Ac as described in section 2.2.5.1 (Assay 2), the purified sialic acids were divided equally into four aliquots. The first aliquot (positive control) was incubated in PBS, pH 7.0 at 37 °C for 2 hours. The other three aliquots were incubated in the presence of 0.1 M NaOH (37 °C for 30 min), influenza C/JJ/50 virus and sialate-pyruvate-lyase, respectively, as described previously (section 2.2.4.4-2.2.4.6). All samples, together with authentic sialic acid standards, were analyzed by fluorometric HPLC. The eluates were collected (1 mL/tube) and the radioactivity of each fraction was determined by scintillation counting. The nature of the radiolabeled products was identified by correlating the elution time to those of the authentic sialic acid standards. Negative control was afforded by incubating microsomal proteins with [³H]AcCoA in the absence of CMP-Neu5Ac.

2.2.6.3 *Separation and Identification of CMP-Neu5,9Ac₂*

The separation and identification of CMP-Neu5,9Ac₂ as the enzyme product was performed using essentially the same enzyme assay, with some modifications, as that described in section 2.2.5.1 (Assay 2). The reaction was initiated by the addition of 0.25 μCi [³H]AcCoA. After incubation for 15 min at 37 °C, the reaction was chased with 0.8 μL of 0.3 mM AcCoA and incubated for a further 15 min. The reaction was terminated by the addition of ice-cold ethanol to a final concentration of 90 %. The sample was kept on ice for 1 h, following which insoluble materials were removed by centrifugation at 14 000 rpm for 30 min. The purification of putative CMP-Neu5,9Ac₂ was performed as described by Fritsch *et al.* (1996) [111]. Briefly, the supernatant obtained following centrifugation was lyophilized, re-suspended in 200 μL of water and applied to a DEAE-Sepharose FF column (0.5 mL bed volume, acetate form). The column was then washed with 5 mL of ice-cold water and subsequently both the free and CMP-linked sialic acids were eluted with 5 mL of 70 mM NH₄OAc solution (pH 7.5) followed by a further 5 mL of ice-cold water. The eluate was lyophilized, and the residue was taken up in 15 μL of water. This suspension was then analyzed by radio-TLC on cellulose TLC-plates in ethanol:1M NH₄OAc, pH 7.3 (7:3, by volume).

Additionally, the incubation was carried out using the same procedure described above except that the reaction was stopped by the addition of propionic acid. The enzyme incubation performed in the absence of CMP-Neu5Ac was used as a control.

2.2.6.4 HPTLC Immuno-Overlay Assay of 9-O-Acetylated GD₃

For HPTLC immuno-overlay assay [113], the enzyme assay was carried out under the same condition described in section 2.2.5.1 (Assay 4), except that non-radiolabeled AcCoA was used as the donor substrate. The enzyme products, prior to or after saponification, were applied to a HPTLC silica gel 60 plate and developed in methanol:chloroform:20 mM CaCl₂ (4:5:1, by volume). After being completely dried under a stream of cold air, the plate was plasticized by chromatographing in a freshly prepared hexane solution which was saturated with polyisobutylmethacrylate. Following thorough drying, the plate was immersed in PBS containing 1 % BSA for 30 min at room temperature. The buffer was decanted and the plate was washed twice with PBS. The plate was then overlaid with monoclonal antibody (mAb) against 9-O-acetylated GD₃, and incubated at 4°C overnight. The unbound antibody was washed away in three steps using 0.05 % Tween 20-PBS, each for 5 min under shaking. The chromatogram was incubated with an alkaline phosphatase-labeled secondary antibody from mouse at a dilution of 1:1000 in 1 % BSA-PBS for 60 min at room temperature and washed as above. The plate was visualized with BCIP/NBT reagent as instructed by the manufacturer, and the reaction was stopped by rinsing the plate with distilled water and drying.

2.2.7 Factors Influencing 7(9)-OAT Activity

2.2.7.1 Influences of pH, Temperature, Time and Protein Concentration

The influences of various factors, including pH, temperature, incubation time and protein concentration on 7(9)-OAT activity were investigated using the standard 7(9)-OAT assay outlined in section 2.2.5.1. The time, temperature, pH, as well as the protein, substrate and inhibitor concentrations are indicated in the figures under section 3.2.8, page 54.

2.2.7.2 Determination of the Apparent K_M Values for AcCoA and CMP-Neu5Ac

The apparent K_M for AcCoA was determined using essentially the same procedure as described in section 2.2.5.1. Briefly, 50 µg of microsomal protein and 600 µM CMP-Neu5Ac were incubated together with different concentrations of AcCoA between 0 and 70 µM under optimal conditions (37 °C for 15 min, pH 7.0).

The apparent K_M for CMP-Neu5Ac was determined as above except that the AcCoA concentration was kept constant at 8.3 µM and the concentration CMP-Neu5Ac was varied between 0 and 600 µM. The K_M values were determined from double-reciprocal plots according to the method of Lineweaver-Burk.

2.2.7.3 *Determination of the K_i Value for CoA*

Fifty μg of microsomal membrane protein was incubated together with different concentrations of CoA (0, 10, 15 and 20 μM) at AcCoA concentrations of 8 μM and 16 μM , respectively. The enzyme activity was determined as described in section 2.2.5.1 and the K_i value was determined using the method described in Dixon *et al.* (1958) [114].

2.2.7.4 *Influences of Amino Acid-Modifying Reagents and Potential Inhibitors on 7(9)-OAT Activity*

Utilizing Golgi membrane fractions isolated as described in section 2.2.1.3, page 23. The influences of DDT (1 mM), DEPC (2 mM), PCMB (2 mM) and acetic acid anhydride (2 mM and 10 mM), as well as saponin (0.005-0.10 %, w/v) on 7(9)-OAT activity were investigated. Assays were carried out under optimal conditions (37 °C, pH 7.0 for 15 min) as outlined in section 2.2.5.1.

2.2.8 *Trypsin Treatment of Golgi Vesicles*

Golgi proteins (3 $\mu\text{g}/\mu\text{L}$) were treated with trypsin (protein:trypsin=150:1, w/w) in 50 μL of 20 mM potassium phosphate buffer, pH 7.0, containing 50 mM KCl and 0.25 M sucrose in the presence and absence of 0.5 % Triton X-100, at 25 °C for 10 min [115]. The incubation was terminated by the addition of three volumes of ice-cold buffer containing 2 mg/mL BSA. After centrifugation at 100 000 x g for 30 min, the Golgi vesicles were surface-washed twice with 1.5 mL of ice-cold buffer, re-suspended in the original volume of 50 mM Tris-HCl buffer, pH 7.45, containing 0.25 M sucrose and immediately used for the determination of ST and 7(9)-OAT activity.

2.2.9 *Preloading of Golgi Vesicles with CMP-Neu5Ac*

Intact Golgi vesicles were incubated with 500 μM CMP-Neu5Ac in 50 μL of 50 mM Tris-HCl buffer (pH 7.45), containing 10 mM MgCl_2 and protease inhibitor (CompleteTM) at 30 °C at time points between 5 min and 30min. Three volumes of the same buffer containing 0.25 M sucrose were used to terminate the incubations. These CMP-Neu5Ac-preloaded Golgi vesicles were pelleted (100 000 x g for 30 min), surface-washed with ice-cold PK buffer, and immediately used in 7(9)-OAT activity assays.

In addition, Golgi vesicles were also preloaded with CMP-Neu5Ac in the presence of CMP, CoA, DIDS or KI-8110 under the same condition described above. Golgi vesicles preloaded in such a way were referred to as CMP-Neu5Ac-preloaded Golgi vesicles I.

2.2.10 Incubation of CMP-Neu5Ac-Preloaded Golgi Vesicles with [³H]AcCoA

CMP-Neu5Ac-preloaded Golgi vesicles or CMP-Neu5Ac-preloaded Golgi vesicles I were incubated with [³H]AcCoA at 37 °C in 30 μL of PK buffer containing Complete™ and Pefabloc. The reaction was initiated with the addition of 0.8 μL of [³H]AcCoA (0.25 μCi/μL), and kept at 37 °C for 15 min. After chasing with 0.8 μL of AcCoA (8.3 μM, final) for another 15 min at 37 °C, the reaction was stopped by the addition of propionic acid to a concentration of 2 M. The acid-soluble fraction was separated from the acid-insoluble fraction *via* centrifugation. Radiolabeled sialic acids were released, purified and analyzed by radio-TLC on silica gel plate or cellulose plate. The radioactivity associated with sialic acids was then used for the calculation of enzyme activity.

Additionally, the incubation of CMP-Neu5Ac-preloaded Golgi vesicles with [³H]AcCoA was carried out in the presence of CMP, CoA, DIDS or KI-8110. The enzyme activity was determined using the procedure described above.

2.2.11 The Transfer of [³H]Neu5,9Ac₂ onto Endogenous Glycoconjugates

One hundred and fifty μg of intact Golgi vesicles was incubated with [³H]AcCoA and CMP-Neu5Ac (200 μM) in 30 μL of PK buffer containing 50 mM KCl, protease and esterase inhibitors. The reaction was initiated by the addition of 1 μL of [³H]AcCoA (0.25 μCi/μL) and incubated at 37 °C for 20 min. Following the addition of 1 μL of non-radiolabeled AcCoA to a final concentration of 5.2 μM, the mixture was kept at 37 °C for a further 20 min. In order to inhibit the further transport of CMP-Neu5Ac and AcCoA into the Golgi vesicles, DIDS was added to a final concentration of 1 mM. The suspension was then further incubated at 37 °C between 0 and 4 h, following which the reaction was terminated by the addition of propionic acid.

For all assays carried out in this manner the acid-soluble and acid-insoluble material was separated by centrifugation at 14 000 rpm for 30 min. The resulting pellet was washed three times with 500 μL ice-cold water, and the glycosidically linked sialic acids released by 200 μL 2M propionic acid (80 °C for 4 h) were analyzed by radio-TLC. The acid-soluble

material present in the supernatant was lyophilized and also analyzed by radio-TLC on silica gel plate.

2.2.12 CMP-[³H]Neu5,9Ac₂ as Sialic Acid Donor for STs

2.2.12.1 Preparation of Asialo-Bovine Submandibular Gland Mucin (Asialo-BSM)

Ten mg of BSM was hydrolyzed with 7 mL of 0.1 M HCl at 80 °C for 1 h, and was extensively dialyzed against distilled water at 4 °C [84]. Analysis of the lyophilized retentates for sialic acids content by BIAL-test showed that 60 % of the sialic acids was removed.

2.2.12.2 CMP-[³H]Neu5,9Ac₂ as Donor Substrate for STs

Putative CMP-[³H]Neu5,9Ac₂ was prepared using the same procedure described in Section 2.2.6.3, except that the concentration of the acceptor substrate, CMP-Neu5Ac, was decreased to 300 μM. Following purification using DEAE-Sepharose FF, approximately 26 000 dpm of putative CMP-[³H]Neu5,9Ac₂ was incubated with either 150 μg human colonic Golgi protein or 9 mU of rat liver α2,6-ST (EC.2.4.99.1) as described by Sadler *et al.* (1979) [116]. Asialofetuin (200 μg) and asialo-BSM (87 μg) were used as the ST acceptor substrates.

The reaction was carried out by incubating the appropriate enzyme with CMP-[³H]Neu5,9Ac₂ in 50 μL of 50 mM sodium cacodylate buffer, pH 6.5, Triton X-100 (0.5 or 1 %) and 1 mM Pefabloc at 37 °C for 30 to 120 min. When rat liver α2,6-ST was utilized, 25 μg BSA was added to the mixture. The reaction was terminated by the addition of TCA to a final concentration of 5 %. The radioactivity incorporated into glycoproteins was determined using the procedure described in section 2.2.3.2, page 23.

ST assays for both human colonic Golgi ST and rat liver α2,6-ST were also performed using CMP-[¹⁴C]Neu5Ac (0.1 μCi, 10 nmol) as the donor substrate.

2.2.13 Solubilization of Membrane-Bound 7(9)-OAT

2.2.13.1 Freeze-Thawing

Microsomes (100 μg) in PK buffer containing 50 mM KCl were frozen at -80 °C and thawed at room temperature. Following a second freeze-thawing cycle, the mixture was centrifuged at 100 000 x g for 30 min. The resulting supernatant (freeze sup) and pellet (freeze pellet) were re-suspended in PK buffer, and used for the determination of 7(9)-OAT activity.

2.2.13.2 *Salt Extraction*

The extraction was carried out according to the procedure described by Mancini *et al.* (1999) [117] with some changes. Two hundred μg of microsomal protein was suspended in 50 μL of PK buffer containing 1 M KCl. The mixture was incubated at 4 °C for 30 min. After centrifugation at 100 000 x g for 30 min, the supernatant was removed and set aside, while the pellet (salt pellet) was washed with 50 μL of ice-cold water. The supernatant obtained following centrifugation at 14 000 rpm for 15 min was then combined with the previous supernatant and desalted by ultrafiltration (Centrex 3 KD). The resulting desalted residue (salt sup), as well as the washed pellet, was used in 7(9)-OAT assay.

2.2.13.3 *EDTA Extraction*

Microsomal proteins were extracted with EDTA as described in Lachaal *et al.* (2000) [118]. Briefly, 200 μg of microsomal protein was dispersed in 1 mM EDTA (pH 7.5) in a final volume of 200 μL and incubated on ice for 30 min. The supernatant (1mM EDTA-sup) and pellet (1 mM EDTA-pellet) obtained after centrifugation at 100 000 x g for 30 min were kept on ice until required. A portion of the 1 mM EDTA-pellet was re-suspended in 150 μL of 100 mM EDTA (pH 10.3), immediately centrifuged at 100 000 x g for 30 min, and washed with ice-cold water. The resulting pellet and supernatant were referred to as 100 μM EDTA-pellet and 100 μM EDTA sup, respectively. All the fractions obtained were subsequently used for the determination of 7(9)-OAT activity.

2.2.13.4 *Detergent Solubilization*

Stock solutions (1-10 %) of the detergents (CHAPS, octylglucoside, Triton X-100, Nonidet P40, LUBROL Px and Zwittergent 3-12) were prepared in water. Different volumes of the appropriate detergent stock solution were added by stirring to membrane suspensions to give detergent/protein ratios that varied between 1:10 and 10:1. Generally, 3-5 mg/mL of total membrane protein in PK buffer, containing 50 mM KCl and protease inhibitors was incubated with the detergent at 4 °C for 30 min with occasional shaking. The clear supernatant obtained following centrifugation at 100 000 x g for 30 min was then used to determine 7(9)-OAT activity.

In some experiments, solubilization was also performed in the presence of either 1 mM DTT, 20 % (v/v) glycerol or 300 μM CMP-Neu5Ac.

2.2.13.5 *Preparation of Lecithin Stock Solution*

Lecithin stock solution was prepared using the procedure described in Schägger *et al.* (1990) [119]. Eighteen mg of lecithin was dissolved in 250 mM of PK buffer, pH 7.0, containing 50 mM KCl and 7.5-9.5 % CHAPS. The molar ratio of CHAPS/lipid is 2-3, which is close to the lowest ratio necessary to maintain the lipids in solution. The stock solutions can be regarded as lipid-saturated mixed-detergent micelles.

2.2.14 *Sialate-O-Acetylerase Assay*

Assay 1 [55]: MU-Ac was used as the substrate. Esterase assay was carried out at 37 °C for 10 min in 100 µL of 10 mM Tris-HCl buffer, pH 7.45, containing 200 µM MU-Ac and 5-10 µg of protein sample. The reaction was terminated by the addition of 100 µL of absolute ethanol. The amount of released MU was determined fluorometrically at excitation and emission wavelengths of 365 nm and 460 nm, respectively. One unit of enzyme activity equals to one nmol of MU released per min under the condition used.

Assay 2 [55]: Free *O*-acetylated sialic acid (Neu5,9Ac₂ or Neu5,7(8),9Ac₃) was used as the substrate. Esterase assay was carried out in 100 mM Tris-HCl buffer, pH 7.45, at 37 °C for 15-30 min in the presence of 1 mM of substrate and 50-500 µg of protein. The reaction was stopped by heating at 95 °C for 2 min, and centrifuged at 14 000 rpm for 5 min. The amount of released acetic acid present in the resulting supernatant was determined using a commercially available kit (Boehringer Mannheim, Germany) as instructed by the manufacturer. One unit of enzyme activity equals to 1 nmol of acetic acid released from *O*-acetylated sialic acid per min under the condition used. In order to confirm the esterase activity observed using this assay, the corresponding release of de-*O*-acetylated sialic acids was monitored by HPLC analysis as described in section 2.2.4.7, page 25.

Assay 3: Using BSM as the substrate, esterase assay was carried out essentially as described in Assay 2. The substrate concentration was calculated according to the amount of 9-*O*-acetylated sialic acids present in BSM, which was determined using the BIAL test and HPLC analysis.

3 Results

3.1 Sialic Acid Analysis

3.1.1 Sialic Acids in Human Colonic Cell Lines

Due to the heterogeneity and variability of human colonic tissue samples obtained from patients who underwent surgical resection of colorectal carcinomas, cell lines derived from human colonic epithelium were initially thought to be the most suitable material for the study of sialate-*O*-acetyltransferase. As a basis for investigating the enzymatic reaction/s that governs sialate *O*-acetylation in human colonic mucosa, the nature and quantity of *O*-acetylated sialic acids present in the two human colonic cell lines obtained were analyzed.

Sialic acids were released from cell pellets by mild acid hydrolysis, purified and analyzed by fluorometric HPLC. As shown in Figure 3-1, a significant amount of Neu5Ac (peak 1) was observed. A second peak (peak 2) was also observed, which could be oligo-*O*-acetylated Neu5Ac or a reagent peak. Under the condition used, a reagent peak was always found to overlap with a peak corresponding to oligo-*O*-acetylated Neu5Ac. However, the HPLC chromatograms, before and after the treatment with NaOH, indicate that no *O*-acetylated sialic acids were expressed in the cell lines tested, because *O*-acetylated sialic acids treated in this manner can be always completely de-*O*-acetylated, leading to a reduction in the peak magnitude and corresponding increase in the amount of Neu5Ac. Therefore, Neu5Ac was the only sialic acid detected in the two cell lines investigated.

In certain cancerous cell lines derived from primary human colonic adenocarcinomas, *O*-acetylated sialic acids were also found not to be present [69]. One reason for this phenomenon may be the loss of certain environmental stimuli such as bacterial by-products, which are lost due to the sterile and uniform conditions used in tissue culture. Such stimuli might be essential for the expression of *O*-acetylated sialic acids [69]. Moreover, histological analysis has shown that *O*-acetylated sialic acids in normal human colonic tissue are associated with sialomucins in the upper half of the crypts in the left colon, and the reverse pattern was observed in the right colon [120;121]. Therefore, the selection of cell subpopulations in tissue culture could be another reason that no *O*-acetylated sialic acids were observed in certain cell lines.

Nevertheless, the activity of 7(9)-OAT in one cell line was assayed using CMP-Neu5Ac as acceptor substrate. No enzyme activity was observed in the membrane and cytosolic fractions

prepared from the cell pellets as described in section 2.2.1.4, page 23. These results showed that these cell lines are not suitable for the study of 7(9)-OAT.

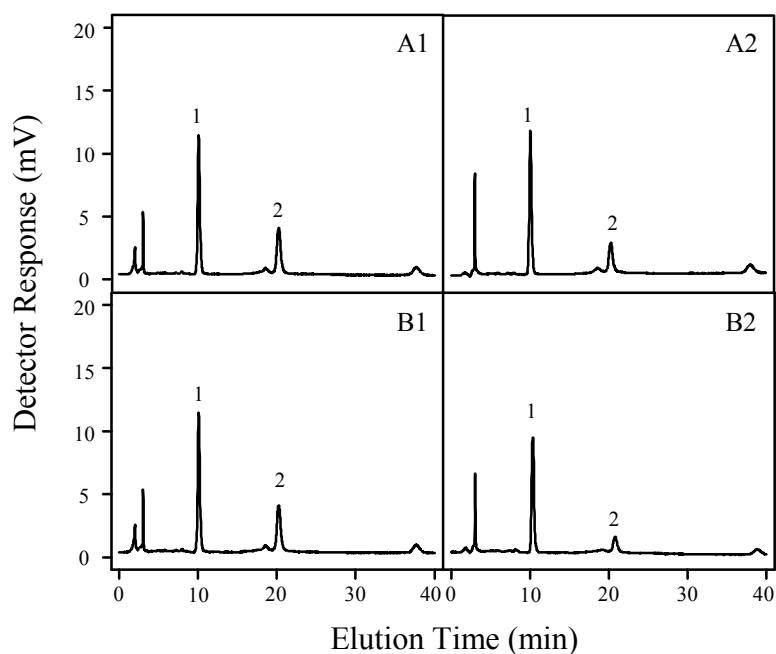


Figure 3-1 Fluorometric HPLC Analysis of Sialic Acids in Human Colonic Cell Lines. Two human colonic cells lines (A-cell line 1; B-cell line 2) were grown under the condition described under section 2.2.4.8, page 26. After harvesting the cells, sialic acids were released by 2 M propionic acid at 80 °C for 4 h. Released sialic acids were collected, purified and analyzed by fluorometric HPLC prior to (1) or after (2) saponification. Peak 1, Neu5Ac; Peak 2, reagent peak.

3.1.2 Nature of Sialic Acids in Human Colonic Mucosa

The nature of sialic acids in different subcellular fractions isolated from human colonic mucosa was investigated by fluorometric HPLC. Glycoconjugate-bound sialic acids were released by mild acid hydrolysis, collected, purified by anion-exchange chromatography and analyzed as described in section 2.2.4, page 24.

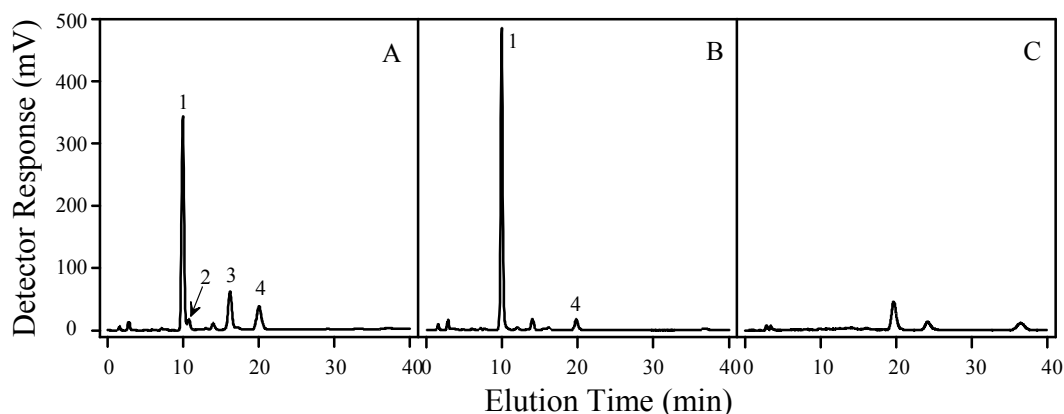


Figure 3-2 Nature of Sialic Acids in the Membrane Fraction from Human Colonic Mucosa.

Glycoconjugate-bound sialic acids in the membrane fraction were released with 2 M propionic acid (80 °C for 4 h), collected *via* ultrafiltration to minimize the loss of *O*-acetyl groups, purified and analyzed by fluorometric HPLC. A, before saponification with 0.1 M NaOH; B, after saponification with 0.1 M NaOH and C, after treatment with sialate-pyruvate-lyase. Peak 1, Neu5Ac; Peak 2, Neu5,7Ac₂; Peak 3, Neu5,9Ac₂; Peak 4, oligo-*O*-acetylated sialic acids/reagent peak.

As can be seen in Figure 3-2A, the sialic acids present in the membrane fraction included Neu5Ac, Neu5,9Ac₂ and oligo-*O*-acetylated sialic acids, as well as a small amount of Neu5,7Ac₂. No Neu5Gc was detected in the uninvolved colonic mucosa. Treatment with 0.1 M NaOH (Figure 3-2B), which can result in the complete de-*O*-acetylation of sialic acids, led to the total saponification of the three peaks corresponding to mono- and oligo-*O*-acetylated Neu5Ac (the latter overlaps with a reagent peak which is not affected by alkaline treatment) with a concomitant increase in Neu5Ac. The incubation of isolated sialic acids with sialate-pyruvate-lyase led to the significant degradation of mono-*O*-acetylated Neu5Ac and Neu5Ac, however, oligo-*O*-acetylated Neu5Ac was only partially degraded (Figure 3-2C). The extent to which oligo-*O*-acetylated Neu5Ac was degraded by sialate-pyruvate-lyase could not be conclusively shown since, as stated above, this peak overlaps with a reagent peak. Additional experiments have shown that oligo-*O*-acetylation of sialic acids hinders the action of sialate-pyruvate-lyase. The similar pattern of sialic acids, for both glycosidically linked (Figure 3-3A) or free (Figure 3-3B), was also found in the cytosolic fraction.

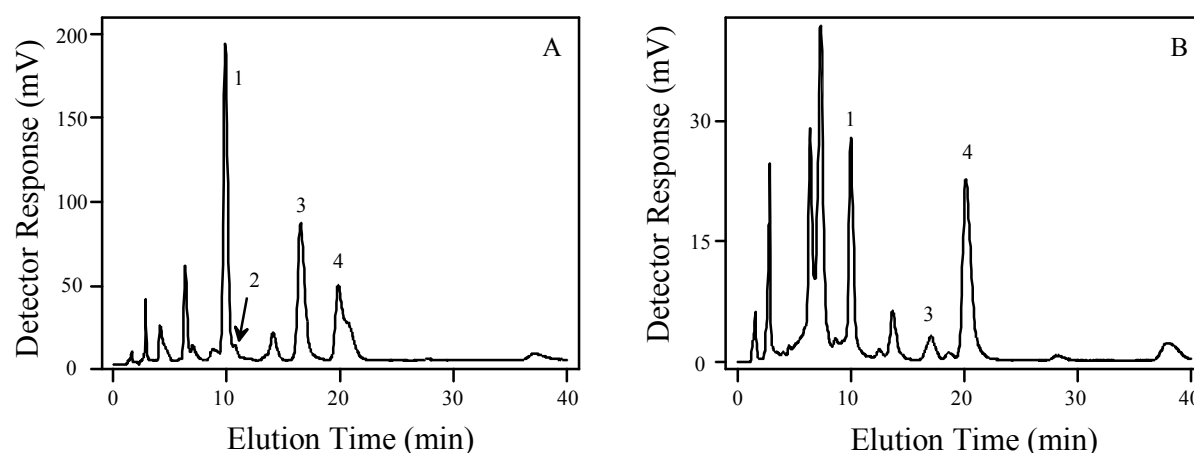


Figure 3-3 Nature of Sialic Acids in the Cytosolic Fraction from Human Colonic Mucosa. Glycosidically linked sialic acids in the cytosolic fraction (A) were released with 2 M propionic acid (80 °C for 4 h) and collected, while free sialic acids (B) in the cytosolic fraction were directly collected. The sialic acids were purified and analyzed by fluorometric HPLC. Peak 1, Neu5Ac; Peak 2, Neu5,7Ac₂; Peak 3, Neu5,9Ac₂; Peak 4, oligo-*O*-acetylated sialic acids/reagent peak.

3.1.3 GC-MS Confirmation of Sialic Acids Present in Human Colonic Mucosa

To directly confirm the nature of sialic acids present in the cytosolic and membrane fractions observed by HPLC, GC-MS analyses were carried out. Figure 3-4 gives an example of the total mass profile of sialic acids in the microsomes prepared from the uninvolved colonic mucosa.

The sialic acids observed using fluorometric HPLC and GC-MS were in good agreement (summarized in Table 3-1). Both Neu5,9Ac₂ and Neu5,8,9Ac₃ were found in the glycosidically linked sialic acids present in the cytosolic and membrane fractions, while Neu5,9Ac₂ was the only free *O*-acetylated sialic acids observed in the cytosolic fraction. However, Neu5,7Ac₂ detected by fluorometric HPLC was not observed by GC-MS analysis. It is unclear why Neu5,7Ac₂ was not detected by GC-MS analysis. One possible explanation may be due to the propensity of acetyl groups present at C-7 of sialic acid to migrate to C-9 [9].

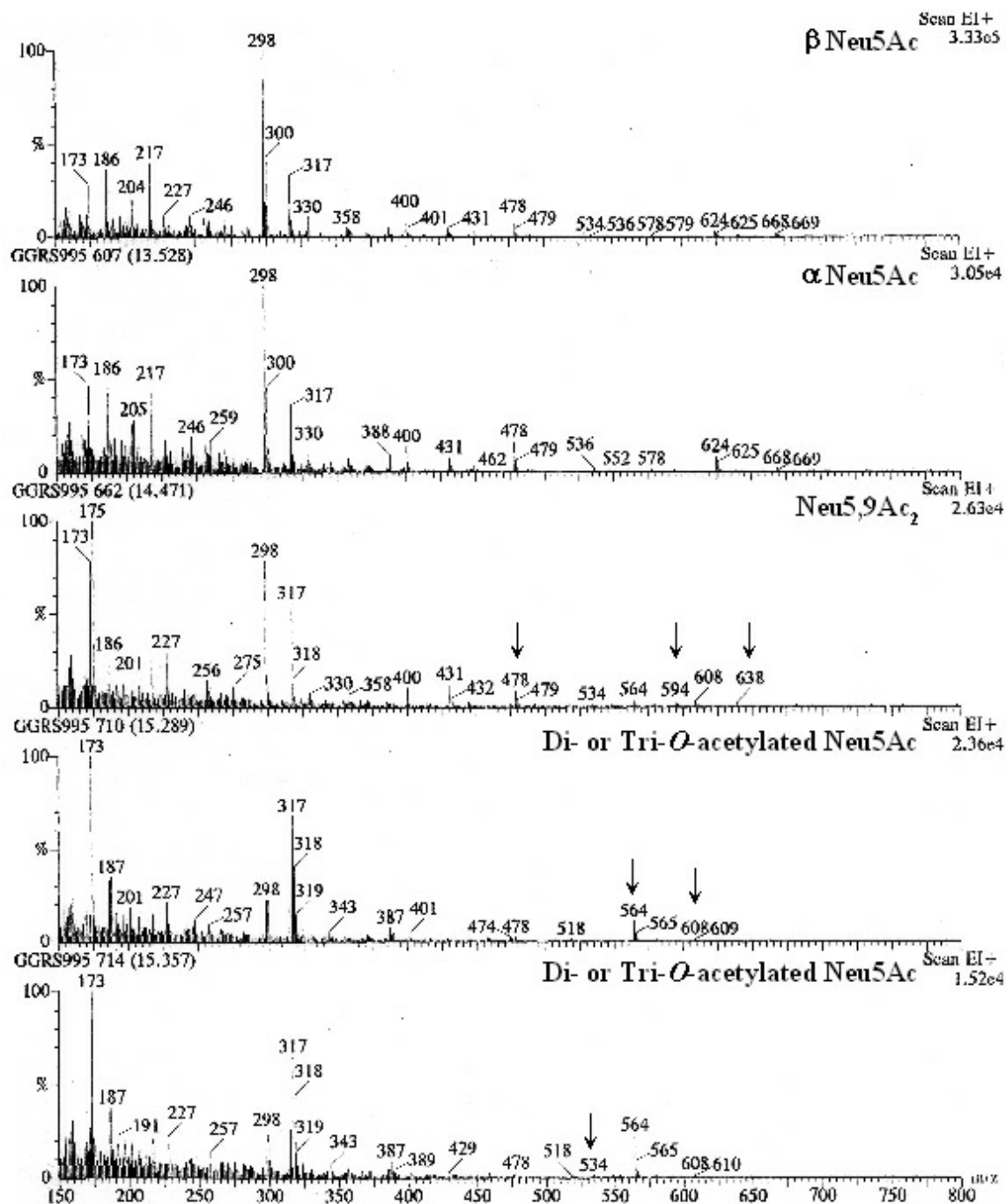


Figure 3-4 Mass Spectrometric Analyses of Microsomal Sialic Acids from Human Colonic Mucosa. The purified sialic acids were converted to their pertrimethylsilylated ester/ether derivatives and applied to a GC-system coupled with EI-MS and analyzed according to a fragmentation scheme by Kamerling *et al.* (1975) [110]. The characteristic fragments of individual sialic acids are indicated by arrows.

Table 3-1 Nature of Sialic Acids Present in Human Colonic Mucosa. Purified glycosidically linked or free sialic acids in the cytosolic or the microsomal fractions were analyzed by fluorometric HPLC and GC-MS as described in section 2.2.4, page 24.

Subcellular fractions	Fluorometric HPLC	GC-MS
Cytosol	Neu5Ac	Neu5Ac
	Neu5,7Ac ₂	Neu5,9Ac ₂
	Neu5,9Ac ₂	Neu5,8,9Ac ₃
	Oligo- <i>O</i> -acetylated Neu5Ac	
Microsomes	Neu5Ac	Neu5Ac
	Neu5,7Ac ₂	Neu5,9Ac ₂
	Neu5,9Ac ₂	Neu5,8,9Ac ₃
	Oligo- <i>O</i> -acetylated Neu5Ac	Neu5,7,8,9Ac ₄
Free sialic acids in cytosol	Neu5Ac	Neu5Ac
	Neu5,9Ac ₂	Neu5,9Ac ₂

3.2 The Sialate-7(9)-*O*-Acetyltransferase in Human Colonic Mucosa

3.2.1 Substrate Specificity

The 7(9)-OAT activities in the microsomal and cytosolic fractions were assayed as described in sections 2.2.5, page 26, against various exogenous substances including EBNS (96 µg sialic acid), de-*O*-acetylated HCM, CMP-Neu5Ac (600 µM) and Neu5Ac (600 µM), as well as endogenous sialic acid-acceptors. In a standard enzyme assay, the microsomal or the cytosolic protein was incubated, in the presence and absence of a potential exogenous acceptor substrate, with [³H]AcCoA, the donor substrate for 7(9)-OAT [33;34]. In the assay procedure reported here, 2 M propionic acid (pH 2.3) was used instead of other frequently used acids, such as PCA [37], to stop the enzyme reaction. The benefit of this method was that glycosidically linked *O*-acetylated sialic acids can be directly released following heating without the need for a protein precipitation step, which therefore significantly reduces not only the loss of *O*-acetyl groups, but also the time required to perform the assay. Similarly, 2 M propionic acid was also used to stop incubations performed in the presence of CMP-Neu5Ac. This resulted in the hydrolysis of CMP-linked sialic acids, thus allowing sialic acids

to be easily purified by ion-exchange chromatography on DOWEX 2 X 8. The hydrolysis of CMP-Neu5Ac using 0.1 M HCl was not performed, since not only does this result in a significant loss of *O*-acetyl esters [76], but also the Cl⁻ formed cannot be removed by lyophilization. The latter is important, if the direct analysis of un-purified sialic acids by TLC is required, since the effectiveness of TLC is considerably affected by the presence of salts.

Enzyme assay was also carried out using 600 μM of sialyllactose (α2,3- or α2,6-linked) as exogenous acceptors. The reaction was stopped by heating at 95 °C for 5 min instead of propionic acid, and the enzyme products were analyzed by radio-TLC. Moreover, GD₃ (600 μM) was tested as a potential substrate for the 7(9)-OAT, and the products were assayed by either radio-TLC or HPTLC immuno-overlay.

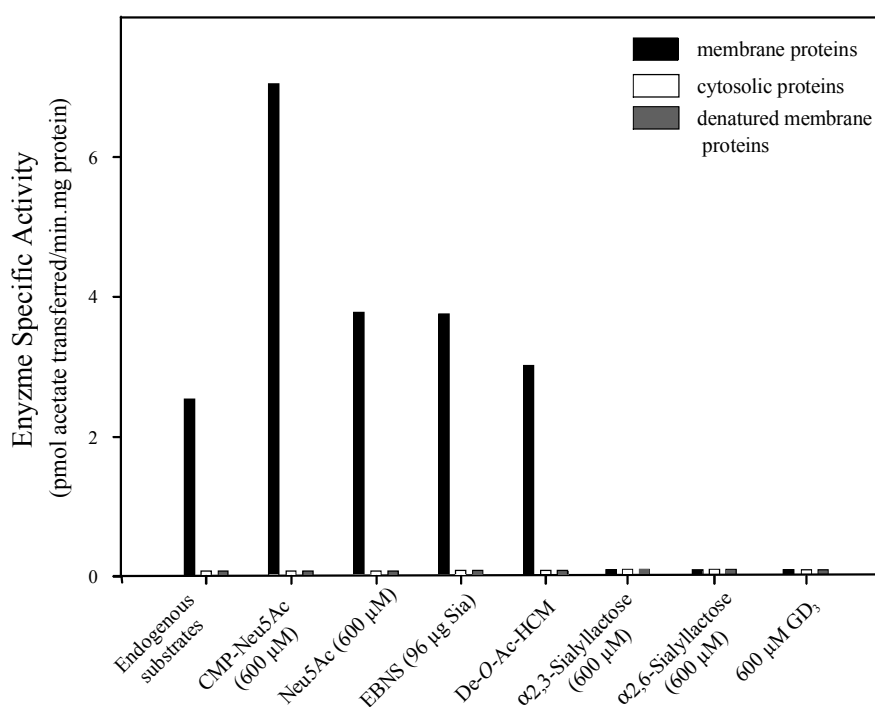


Figure 3-5 Sialate-7(9)-OAT Activity Against Different Potential Acceptor Substrates. Fifty μg of microsomal membrane protein (black bar), cytosolic protein (white bar) or heating-denatured protein (gray bar), prepared from human colonic mucosa, was incubated with [³H]AcCoA under optimal conditions (see Figure 3-17, page 55) in the presence and absence of a number of possible exogenous acceptor substrates. The products of the reaction were purified and the 7(9)-OAT activity was determined using the procedure described in section 2.2.5, page 26. In the case that sialyllactoses and GD₃ were used as exogenous substrates, the endogenous glycoconjugates were removed by precipitation after the enzyme incubation, and only the supernatants were analyzed. One unit of 7(9)-OAT activity is defined as the amount of enzyme that will transfer 1 pmol acetate from AcCoA to sialic acids per minute at 37 °C, pH 7.0.

As can be seen in Figure 3-5, no enzyme activity was observed in the cytosolic fraction, neither using exogenous nor endogenous sialic acid-acceptor substrates, while the incorporation of [³H]acetyl groups, into endogenous sialic acids and a number of exogenous acceptor substrates, was observed when the membrane proteins were used. Furthermore, the incubation of heat-denatured membrane fractions with [³H]AcCoA in the presence and absence of various acceptor substrates did not result in the incorporation of radiolabeled acetate (Figure 3-5). This suggests that the incorporation of radiolabeled acetyl groups onto endogenous and exogenous acceptor substrates is occurring enzymatically, probably *via* the action of a sialate-7(9)-OAT present in the microsomal fraction.

Of all the exogenous acceptor substrates tested, upon the incubation with [³H]AcCoA and the microsomal membrane proteins, the incorporation rate of [³H]acetyl groups into CMP-Neu5Ac is higher than that observed into other exogenous and endogenous substrates (Figure 3-5). The 7(9)-OAT activity observed against endogenous substrates is probably due to the presence of residual endogenous CMP-Neu5Ac in the isolated microsomal pellet fractions. That is, endogenous CMP-Neu5Ac following enzymatic *O*-acetylation would not only be detected following assays using exogenous Neu5Ac and CMP-Neu5Ac as acceptors, but also when performing assays using sialoglycoconjugates, such as EBNS and de-*O*-acetylated HCM as substrates. In the latter case, however, Neu5,9Ac₂ would be transferred from newly formed CMP-Neu5,9Ac₂ to endogenous glycoconjugate acceptors *via* the action of an endogenous sialyltransferase, resulting in the formation of acid-insoluble products which could be detected using the assay system employed.

When GD₃ and sialyllactoses were used as the substrate, the enzyme reactions were stopped by either organic solvent or heating. Under such conditions, the radioactive incorporation into sialic acids linked to endogenous glycoconjugates can not be released. Therefore, no 7(9)-OAT activity against endogenous substrate were detected in these cases (Figure 3-5).

De-*O*-acetylated HCM was a poor acceptor substrate for the membrane-bound sialate-7(9)-OAT, even though HCMs secreted by the human colonic mucosa are highly *O*-acetylated *in vivo* [93;94;103]. Similarly, EBNS and free Neu5Ac were also poor substrates, with the incorporation of [³H]acetate being only slightly increased over that observed for endogenous substrates. No enzyme activity against sialyllactose and GD₃ was observed. Therefore, CMP-Neu5Ac was used as the exogenous substrate in further characterization studies.

3.2.2 Identification of Neo-Synthesized *O*-Acetylated Sialic Acids by Radio-TLC

The nature of *O*-acetylated sialic acids formed was identified, following mild acid hydrolysis of the resulting enzyme products and sialic acid purification, using radio-TLC on silica gel 60 HPTLC plates or cellulose plates.

Figure 3-6 provides an example of the confirmation of *O*-acetylated sialic acids formed by radio TLC on silica gel plates. The incubation of membrane proteins in the absence of CMP-Neu5Ac resulted in a low level of incorporated radioactivity (Figure 3-6C). However, as can be seen in Figure 3-6A, two radioactive peaks were observed in the presence of CMP-Neu5Ac, one of which (peak 1) co-migrated with Fe^{3+} /orcinol/HCl-stained authentic Neu5,9Ac₂ and the other (peak 2) with oligo-*O*-acetylated sialic acids. These radioactive peaks can be saponified by an overnight incubation in the presence of ammonium vapor (Figure 3-6B), suggesting that radioactive ester groups had been formed upon incubation of membrane proteins with [³H]AcCoA.

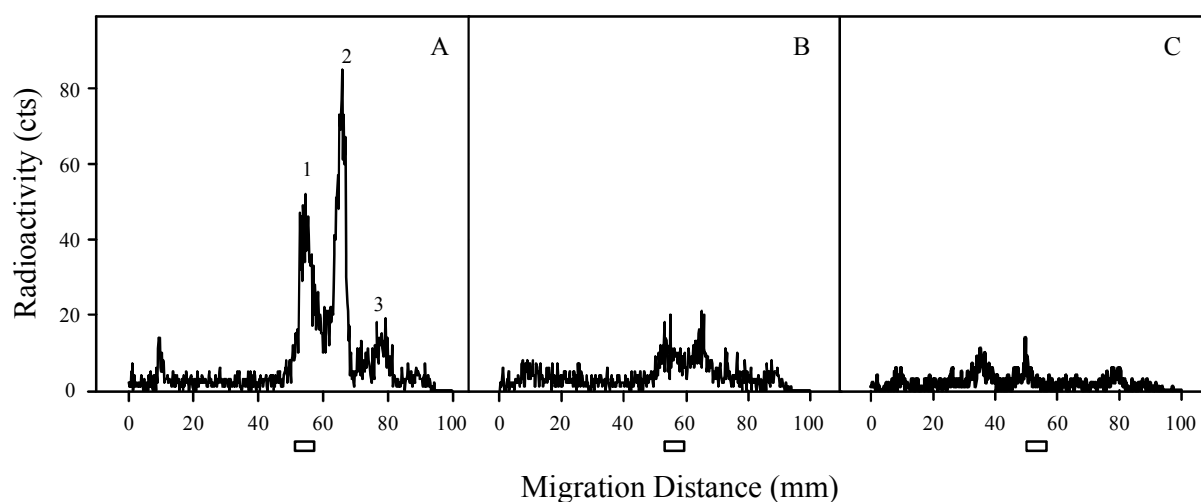


Figure 3-6 Radio-TLC Analysis of Purified *O*-Acetylated Sialic Acids on HPTLC Silica Gel Plate.

Following the incubation of microsomal membrane protein (50 μg) with [³H]AcCoA and 600 μM CMP-Neu5Ac, the enzyme products were hydrolyzed, purified and analyzed by radio-TLC on silica gel plates, which was developed in methanol:chloroform:20 mM CaCl_2 (5:4:1, v/v/v). Radiolabeled sialic acids, before (A) and after (B) saponification, were analyzed using an automatic TLC-linear analyzer. Control (C) incubation was performed in the absence of CMP-Neu5Ac. The migration distance of Fe^{3+} /orcinol/HCl reagent-stained authentic Neu5,9Ac₂ is indicated. Peak 1, Neu5,9Ac₂; Peak 2, oligo-*O*-acetylated sialic acids; 3, acetic acid.

The radio-TLC performed on cellulose plates also verifies the formation of *O*-acetylated Neu5Ac following the incubation of microsomal protein with [³H]AcCoA and CMP-Neu5Ac (Figure 3-7).

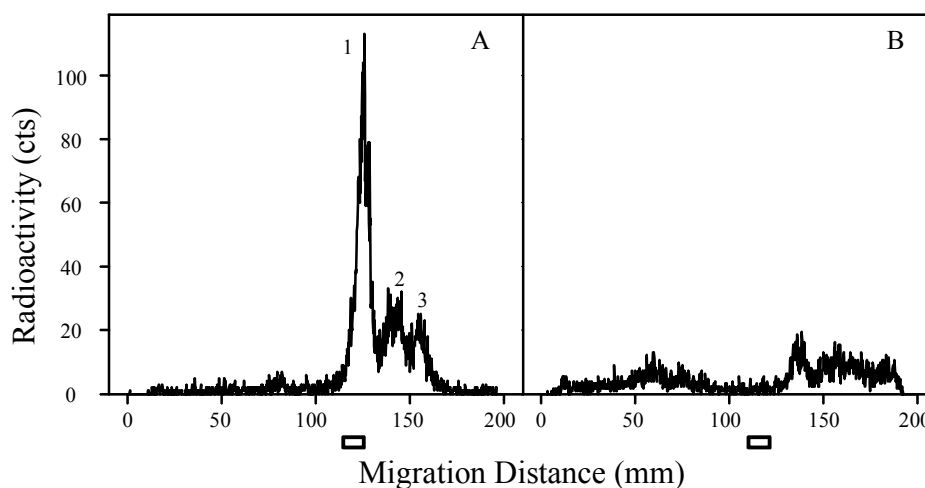


Figure 3-7 Radio-TLC Analysis of Purified *O*-Acetylated Sialic Acids on Cellulose Plate. Following the incubation of microsomal membrane protein (50 μ g) with [³H]AcCoA and 600 μ M CMP-Neu5Ac, the enzyme products were hydrolyzed, purified and analyzed by TLC on cellulose plates. TLC plates were developed in butanol:propanol:0.1 M HCl (1:2:1, v/v/v). Radiolabeled sialic acids were analyzed using an automatic TLC-linear analyzer. Control (B) incubation was performed using heating-denatured proteins. The migration distance of Fe³⁺/orcinol/HCl reagent-stained authentic Neu5,9Ac₂ is indicated. Peak 1, Neu5,9Ac₂; Peak 2, oligo-*O*-acetylated sialic acids; 3, acetic acid.

3.2.3 Identification of Neo-Synthesized *O*-Acetylated Sialic Acids by Radio-HPLC

In order to further confirm the nature of *O*-acetylated sialic acids being formed, HPLC analysis was performed. Fluorometric HPLC affords a better separation of sialic acids, and is faster and more specific than radio-TLC. Therefore, its use, in conjunction with the sialate-9-*O*-acetyl-specific esterase (sialate-9-*O*-acylesterase) of influenza C virus and the sialate-pyruvate-lyase, as well as alkaline treatment, was adopted in order to confirm the formation of *O*-acetylated sialic acid.

Figure 3-8A shows that a number of radioactive peaks (1-3) were observed, however, only two of these possessed retention times characteristic for *O*-acetylated sialic acids. The retention time for peak 2 corresponded with that of authentic Neu5,9Ac₂, while the retention time for peak 3 corresponded to that of authentic oligo-*O*-acetylated Neu5Ac (Figure 3-8F).

The other observed radioactive peak (peak 1), however, seems not to result from the action of sialate-7(9)-OAT, since the same peak was also observed by HPLC analysis of a control sample, in which [^3H]AcCoA was incubated with microsomal proteins in the absence of CMP-Neu5Ac (Figure 3-8E). Radioactive peak 1 is probably the result of the degradation of [^3H]AcCoA to free acetate, with this peak previously been identified in our laboratory, by HPLC analyses, as being free acetate (unpublished data).

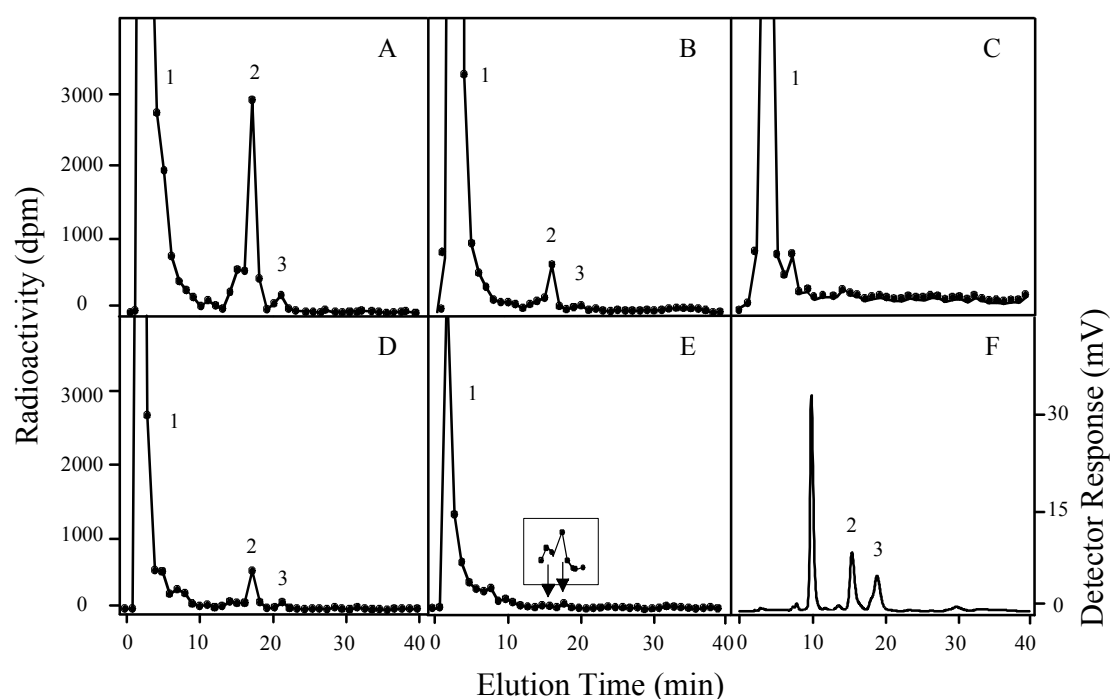


Figure 3-8 Radio-HPLC Analyses of Purified *O*-Acetylated Sialic Acids. Following the incubation of microsomal protein with [^3H]AcCoA and CMP-Neu5Ac, the resulting enzyme products were hydrolyzed, purified and incubated with: A, PBS (pH 7.0) at 37 °C for 2 h; B, sialate-pyruvate-lyase at 37 °C for 2 h; C, 0.1 M NaOH at 37 °C for 30 min and D, influenza C virus at 37 °C for 1 h. Purified sialic acids treated in this manner, together with authentic BSM sialic acid standards, were analyzed by fluorometric HPLC. Fractions of 1.0 mL were collected and radioactivity determined *via* scintillation counting. Control was afforded by the incubation of microsomal membrane protein with [^3H]AcCoA in the absence of CMP-Neu5Ac (E). The elution of BSM sialic acid standards was monitored fluorometrically as described in section 2.2.4.7, page 25. Peak 1, acetic acid; Peak 2, Neu5,9Ac₂; Peak 3, oligo-*O*-acetylated Neu5Ac.

As can be further seen in Figure 3-8E (insert), a small amount of radiolabeled product corresponding to peaks 2 and 3 was also observed in the control incubation. These products are almost certainly due to the presence in the microsomal fraction of a low molecular weight

endogenous substrate, probably CMP-Neu5Ac, which was capable of acting as an acceptor for the 7(9)-OAT.

In further investigations, the incubation of purified sialic acids, prior to HPLC, with sialate-pyruvate-lyase resulted in over 90 % of the radioactivity associated with peak 2 being lost (Figure 3-8B). The complete saponification of peaks 2 and 3 was achieved by pre-incubating sialic acid samples in 0.1 M NaOH prior to HPLC analyses (Figure 3-8C), suggesting that the incorporated radiolabel was primarily occurring as *O*-acetyl esters. In a further experiment, purified sialic acids were incubated with influenza C virus, prior to radio-HPLC (Figure 3-8D). This resulted in the loss of 82 % of the radioactivity associated with peak 2, however, no other peaks were affected following the treatment with influenza C virus. Taken together, these results strongly indicate that the main *O*-acetylated sialic acids formed following the incubation of the membrane fraction prepared from human colonic mucosa with [³H]AcCoA and CMP-Neu5Ac was Neu5,9Ac₂, with a significant amount of oligo-*O*-acetylated sialic acids also being formed.

3.2.4 Evidences that Free CMP-Neu5Ac Acts as the Acceptor Substrate for 7(9)-OAT

The stability of CMP-Neu5Ac under the condition used for enzyme assay was checked by TLC analysis on silica gel plates. In comparison with the standard (Figure 3-9, lane 4), CMP-Neu5Ac was not hydrolyzed after being incubated with PK buffer containing 50 mM KCl, pH 7.0 at 37 °C for 15 min (Figure 3-9B, lane 1). However, the majority of CMP-Neu5Ac was hydrolyzed to CMP and Neu5Ac following an incubation in the presence of 2 M propionic acid at 80 °C for 15 min (Figure 3-9, lane 2) or heating at 95 °C for 5 min (Figure 3-9, lane 3).

The TLC analysis of CMP-Neu5Ac used in 7(9)-OAT activity determinations revealed the existence of a contaminant that could be stained with Fe³⁺/orcinol/HCl reagent. This contaminant was found to co-migrate on silica-TLC with authentic Neu2en5Ac (Figure 3-9, Lane 7). Since Neu2en5,9Ac₂ has been found to occur in nature [122], the possibility that Neu2en5Ac could act as an acceptor substrate was investigated. However, no activity was detected when Neu2en5Ac was incubated together with [³H]AcCoA and microsomes (Figure 3-10). Furthermore, substrate specificity studies also showed that Neu5Ac was not as effective an acceptor substrate as CMP-Neu5Ac (Figure 3-5). These results suggest that the degradation product of CMP-Neu5Ac, primarily Neu5Ac, as well as Neu2en5Ac, are not

acting as the acceptors for 7(9)-OAT when CMP-Neu5Ac is being used to determine enzyme activity.

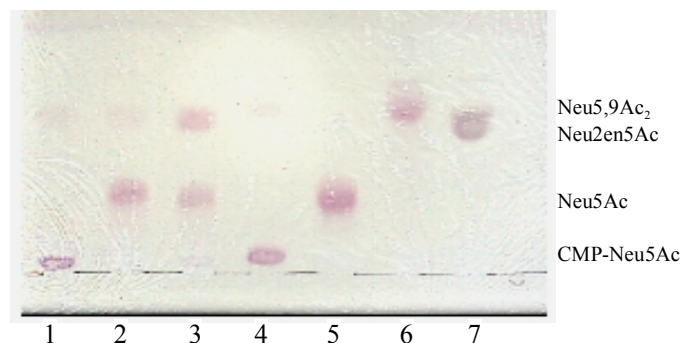


Figure 3-9: Stability of CMP-Neu5Ac under the Condition for Enzyme Assay. Analysis of CMP-Neu5Ac was carried out on a silica gel plate following Lane 1-incubation at 37 °C for 15 min in PK buffer containing 50 mM KCl; Lane 2-incubation with 2 M propionic acid at 80 °C for 15 min; Lane 3-incubation at 95 °C for 5 min in PK buffer containing 50 mM KCl; Lane 4, 5, 6, 7-standard CMP-Neu5Ac, Neu2en5Ac and Neu5,9Ac₂ standard in PK buffer containing 50 mM KCl, respectively. The silica gel plate was developed in methanol:chloroform:20 mM CaCl₂ (5:4:1, v/v/v), and the sialic acids were stained with BIAL reagent.

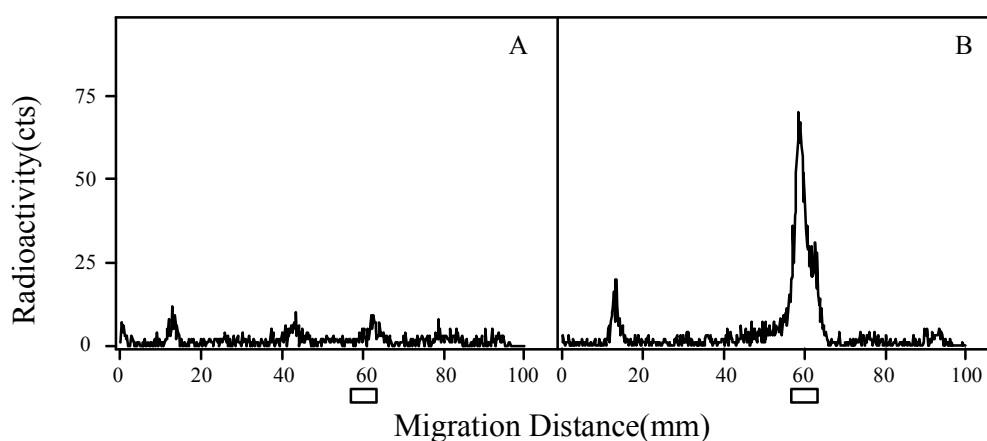


Figure 3-10 Utilizing Neu2en5Ac as the Potential Exogenous Substrate for Colonic 7(9)-OAT. Following the incubation of microsomal protein (50 µg) with [³H]AcCoA, using either 300 µM Neu2en5Ac (A) or 600 µM CMP-Neu5Ac (B) as the exogenous acceptor substrate, the resulting enzyme products were hydrolyzed, purified, and analyzed by TLC on silica gel plates (methanol:chloroform:20 mM CaCl₂, 5:4:1, v/v/v). The migration distance of Fe³⁺/orcinol/HCl reagent-stained authentic Neu5,9Ac₂ is indicated.

In order to further demonstrate that CMP-Neu5Ac can act as the acceptor substrate for the sialate-7(9)-OAT, additional investigations were undertaken to isolate the putative product, CMP-Neu5,9Ac₂, resulting from the incubation of microsomal proteins with [³H]AcCoA and CMP-Neu5Ac. For this purpose, enzyme incubations were stopped with ethanol, following which the enzyme products were isolated by ion-exchange chromatography on DEAE Sepharose FF.

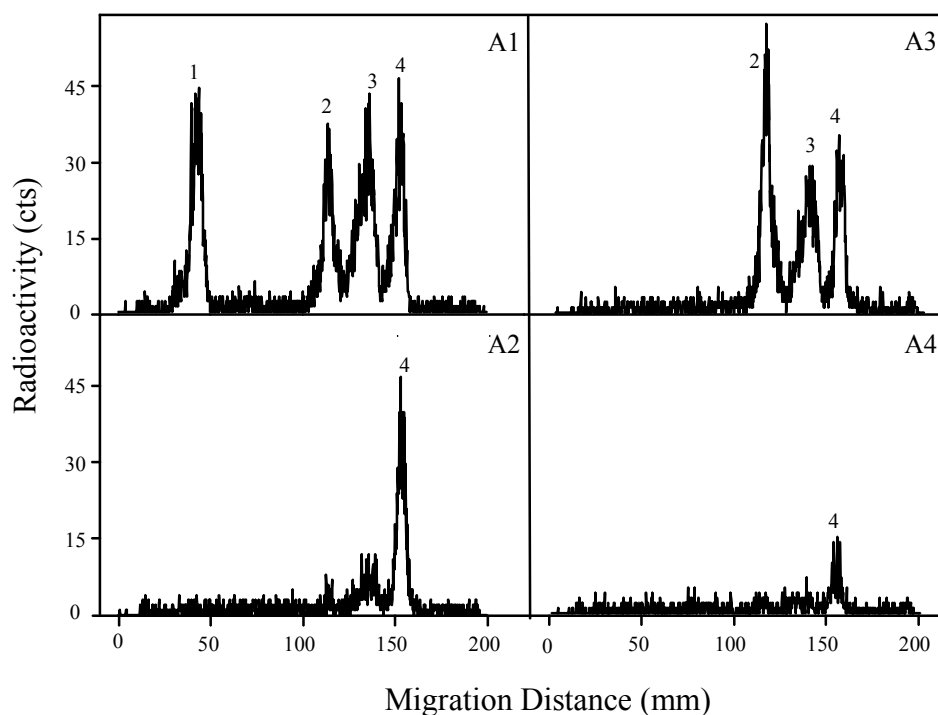


Figure 3-11 The Separation and Analysis of CMP-Neu5,9Ac₂ by Radio-TLC on Cellulose Plates. Microsomal protein was incubated with AcCoA and CMP-Neu5Ac using the standard enzyme assay described in section 2.2.5.1, page 26. The standard enzyme reaction used to produce CMP-Neu5,9Ac₂ was stopped using either ethanol, to avoid product hydrolysis (A1 and A2) or propionic acid, to promote product hydrolysis (A3 and A4). The enzyme products isolated by ion exchange chromatography were then applied to cellulose plates and developed in ethanol:1 M NH₄OAc, pH 7.3 (7:3, v/v). Controls were provided by the incubation of microsomal protein with AcCoA in the absence of CMP-Neu5Ac (A2 and A4). Peak 1, CMP-Neu5,9Ac₂; Peak 2, Neu5,9Ac₂; Peak 3, oligo-*O*-acetylated Neu5Ac; 4, free acetate.

As can be seen in Figure 3-11, four radioactive peaks (1-4) were observed on radio-TLC, while only radiolabeled peak 4 was observed following the incubation performed in the absence of CMP-Neu5Ac (control). Peaks 2 and 3 were identified as Neu5,9Ac₂ and oligo-*O*-acetylated Neu5Ac, respectively, since they co-migrated with the corresponding authentic

standards. Peak 4 is free acetate. The identification of peak 1 as being CMP-Neu5,9Ac₂ was afforded by radio-TLC analysis of the isolated products after stopping the enzyme reaction with propionic acid instead of ethanol. Radio-TLC analysis of enzyme products isolated in this manner resulted in the complete disappearance of peak 1 with a corresponding increase in the radioactivity associated with peak 2 (Neu5,9Ac₂) (Figure 3-11A3), suggesting that CMP-Neu5,9Ac₂ was hydrolyzed by propionic acid to produce Neu5,9Ac₂ and CMP. Again, only radioactive peak 4 was observed for the corresponding control incubations performed in the absence of CMP-Neu5Ac (Figure 3-11A4).

Furthermore, all radioactive peaks observed in Figure 3-11 were susceptible to alkaline treatment with ammonium vapor (Figure 3-12B1-4). The radioactivity associated with peak 4 also disappeared since acetic acid, being volatile, is easily removed when TLC plates are dried, following ammonium treatment, under a stream of cold air.

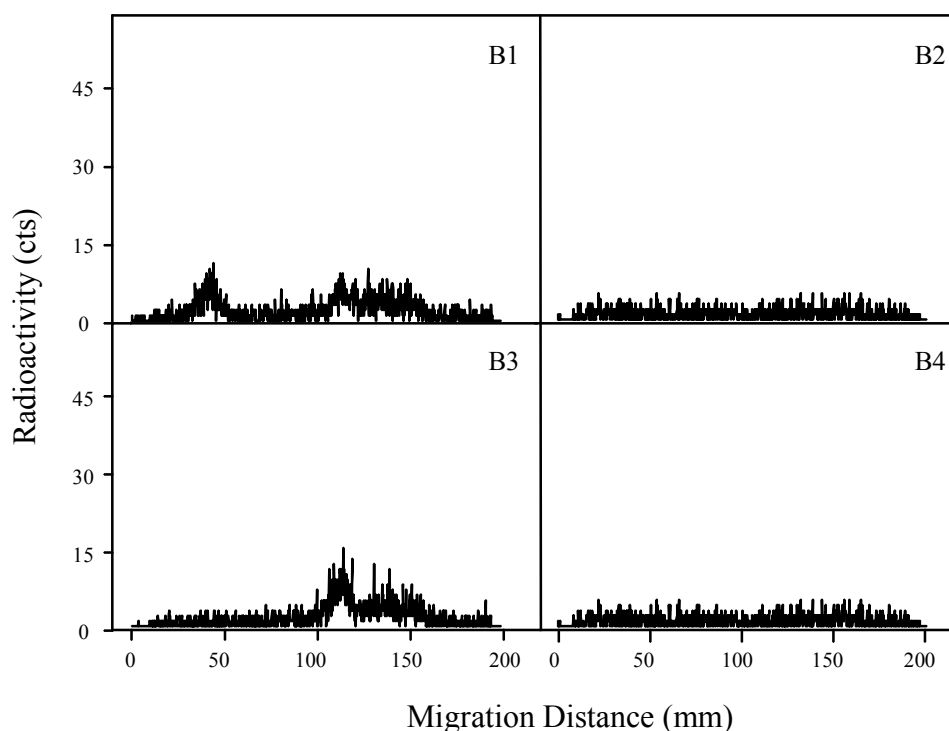


Figure 3-12 Saponification of *O*-Acetylated Sialic Acids. Saponification of the radioactive peaks observed in Figure 3-11 was fulfilled by incubating the TLC plate in a tightly sealed chamber saturated with NH₃ vapor overnight. After dried with a stream of cold air, the plate was analyzed again by an automatic TLC-linear analyzer.

3.2.5 Radiolabeled-CMP-Neu5,9Ac₂ as Sialic Acid Donor for ST

To conclusively demonstrate that CMP-Neu5,9Ac₂ was formed by the action of a membrane-bound 7(9)-OAT in the presence of CMP-Neu5Ac and AcCoA, putative radiolabeled CMP-Neu5,9Ac₂ isolated by ion exchange chromatography was used as the donor substrate for ST. Two ST activities were utilized, the ST activity associated with the Golgi-enriched fraction prepared from human colonic tissues, and the α 2,6-ST from rat liver. As shown in Figure 3-13, the incubation of CMP-[³H]Neu5,9Ac₂ with either rat liver α 2,6-ST (EC. 2.4.99.1) or human colonic Golgi ST resulted in the transfer of [³H]Neu5,9Ac₂ onto asialofetuin. No incorporation of radioactivity into asialofetuin was observed when heat-denatured Golgi ST was used as the enzyme source, while low activity was detected if exogenous substrate was absent.

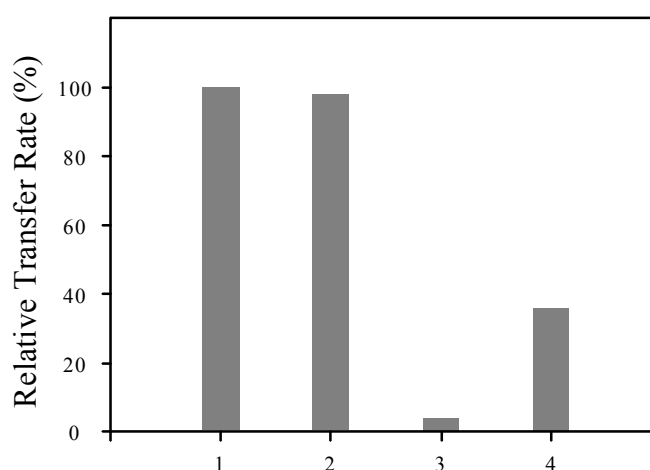


Figure 3-13 Utilization of CMP-[³H]Neu5,9Ac₂ as Sialic Acid Donor for Rat Liver α 2,6-ST and Human Colonic Golgi STs. CMP-[³H]Neu5,9Ac₂ was used as the sialic acid donor and asialofetuin as the acceptor for ST from: 1, rat liver (α 2,6-ST) and 2, Golgi-enriched fraction prepared from human colonic mucosa. The control (3) was afforded by denaturing Golgi protein prior to the determination of ST activity, while control (4) was performed by incubating a Golgi-enriched fraction with CMP-[³H]Neu5,9Ac₂ in the absence of asialofetuin. The rat liver α 2,6-ST activity against CMP-Neu5,9Ac₂ was set at 100%.

3.2.6 Subcellular Localization of the 7(9)-OAT

Using a discontinuous sucrose density gradient, subcellular membranes (fractions A-D) were isolated from human colonic mucosa. ST activity was used as the specific marker enzyme for the Golgi apparatus. In a typical preparation, the highest ST activity was localized in fraction A (Figure 3-14). Using CMP-Neu5Ac as the acceptor substrate, the highest sialate-7(9)-OAT

activity was, likewise, restricted primarily to fraction A, strongly suggesting that the 7(9)-OAT is localized in the Golgi-enriched fraction.

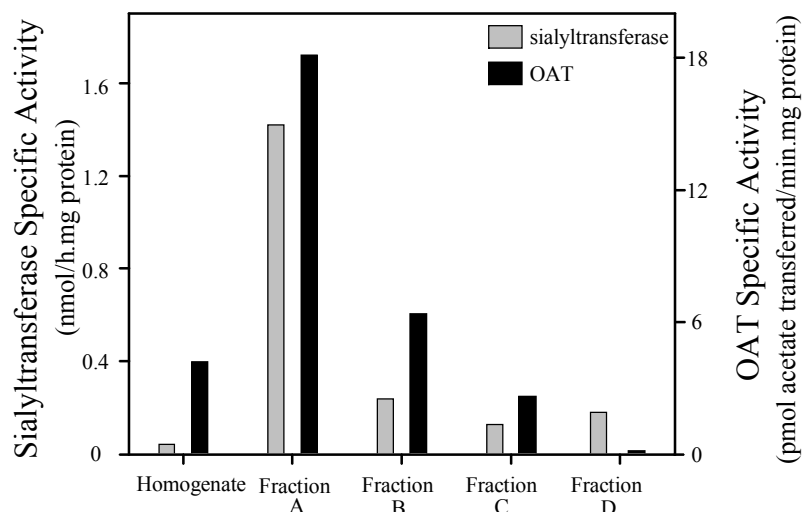


Figure 3-14 Co-localization of ST and 7(9)-OAT Activity in Golgi-enriched Membrane Fraction.

Subcellular membrane fractions were isolated from human colonic mucosa using a discontinuous sucrose density gradient as described in section 2.2.1.3 (procedure 1), page 21. The activity of the Golgi marker enzyme, ST (gray bar), and 7(9)-OAT (black bar) in the crude homogenate, as well as in the different membrane fractions (A-D) were determined. One unit of ST activity is defined as the amount of enzyme that will transfer 1 nmol Neu5Ac from CMP-Neu5Ac to asialofetuin per hour under the experimental conditions used.

3.2.7 Dependence of 7(9)-OAT Activity on the Membrane Integrity

Saponin is a natural steroid that interacts with cholesterol in membranes. At various concentrations, saponin can form 100 nm to 100 μ m wide pores in membranes, through which not only the ions and other small molecules, but also various macromolecules such as colloidal gold-catalase and -albumin can pass [123;124]. Therefore, saponin was used to investigate to what extent 7(9)-OAT activity is dependent on membrane integrity.

As shown in Figure 3-15, the activity of the microsomal 7(9)-OAT was decreased by 20 % and 50 % at a saponin concentration of 0.002 % and 0.01 %, respectively. Total loss of enzyme activity was observed at a saponin concentration of 0.05 %. Likewise, the enzyme activity was decreased by 50 % at 0.01 % saponin when Golgi vesicles were used as the 7(9)-OAT source.

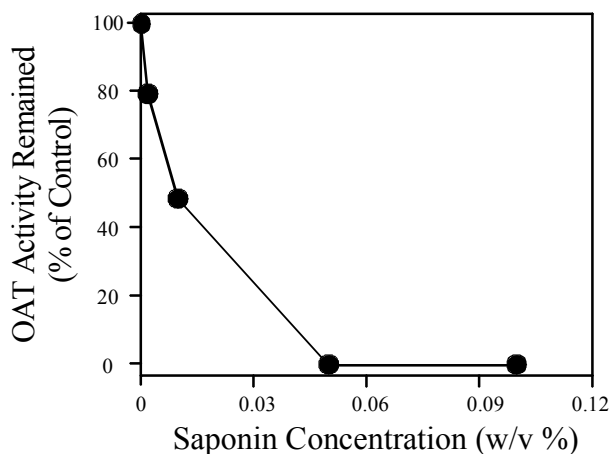


Figure 3-15 The Effect of Saponin on 7(9)-OAT Activity. Using CMP-Neu5Ac as the exogenous substrate, 50 μg of microsomal proteins was incubated with [^3H]AcCoA in the presence of saponin at various concentrations. The enzyme activity was determined using the procedure described in section 2.2.5.1, page 26. The enzyme activity detected in the absence of saponin was set at 100 % (control).

Additionally, the influence of Triton X-100, a non-ionic detergent, on 7(9)-OAT activity was investigated. As shown in Figure 3-16, the enzyme activity was significantly decreased in the presence of Triton X-100 whose concentration is above its critical micellar concentration (about 0.015 %, v/v) [125]. From the results obtained here, it is clear that the activity of 7(9)-OAT is strictly dependent on membrane integrity.

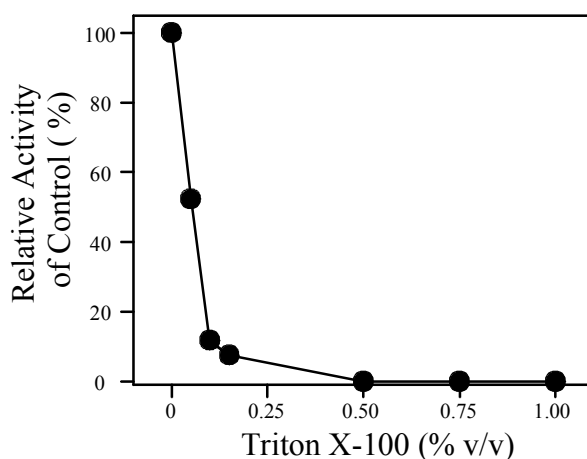


Figure 3-16 The Effect of Triton X-100 on 7(9)-OAT Activity. One hundred μg of microsomal protein was incubated with Triton X-100 at the concentrations indicated. Following a 30 min incubation at 4 $^{\circ}\text{C}$, the suspension was immediately used for the determination of 7(9)-OAT activity using CMP-Neu5Ac as acceptor substrate. The maximal activity detected in the absence of Triton X-100 was set at 100 % (control).

3.2.8 Optimal Conditions for 7(9)-OAT Assay

Using CMP-Neu5Ac as the exogenous substrate, the influences of temperature, pH, time, protein concentration, substrate concentration, and potential inhibitors as well as amino acid-modifying reagents on the activity of 7(9)-OAT were investigated. Microsomes prepared from the same colonic mucosa were used for all investigations. For all parameters investigated, only one factor was varied at any given experiment, with all other factors being kept constant or near their optimal values.

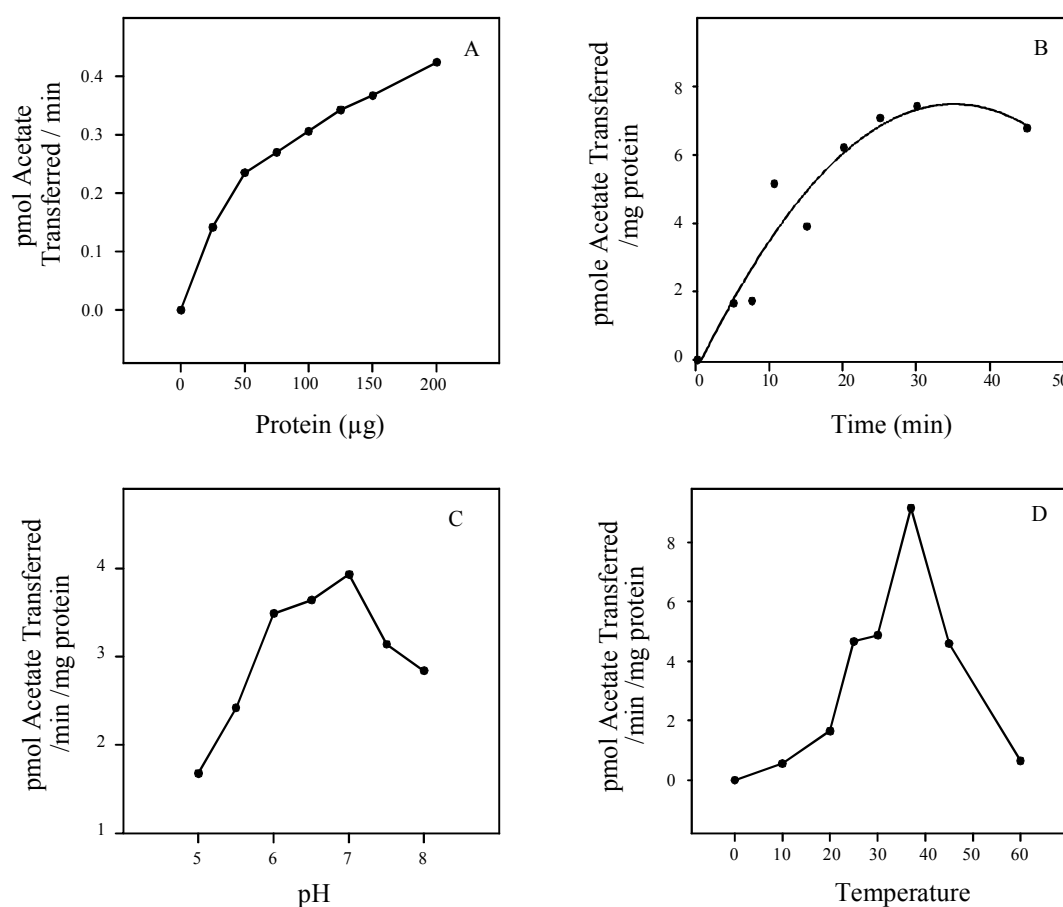


Figure 3-17 Influences of Various Parameters on the Incorporation of [³H]-Acetyl Groups into CMP-Neu5Ac. The influences of protein concentration (A), time (B), pH (C) and temperature (D) on 7(9)-OAT activity were investigated using CMP-Neu5Ac as the acceptor substrate in PK buffer containing 50 mM KCl. Enzyme incubations were performed for panel A at 37 °C, pH 7.0; for 15 min; for panel B at 37 °C, pH 7.0 containing 50 µg protein; for panel C at 37 °C for 15 min containing 50 µg of protein and for panel D at pH 7.0 for 15 min containing 50 µg of protein.

As shown in Figure 3-17, under the experimental conditions used (outlined in the figure legend), the incorporation of [^3H]acetyl groups into CMP-Neu5Ac was linear up to a protein concentration of 50 μg in a final reaction volume of 30 μL (1.7 mg/mL) (Figure 3-17A) and for time points up to 20 min (Fig. 17B). The 7(9)-OAT activity was also found to possess a pH optimum of 7.0 (Figure 3-17C) and a temperature optimum of 37 $^{\circ}\text{C}$ (Figure 3-17D). Therefore, all sialate-7(9)-OAT activity determinations were performed at 37 $^{\circ}\text{C}$ in PK buffer containing 50 mM KCl for 15 min at a protein concentration of 1.7 mg/mL.

3.2.9 Determination of K_M Values for CMP-Neu5Ac and AcCoA

Under optimal conditions, the effects of both donor and acceptor substrate concentrations were studied. The 7(9)-OAT activity was found to be dependent on both the concentrations of AcCoA and CMP-Neu5Ac (Figure 3-18). The apparent K_M value was determined according to the Lineweaver-Burk method. Apparent K_M values of 6.1 μM and 59.2 μM were obtained for AcCoA (Figure 3-18A) and CMP-Neu5Ac (Figure 3-18B), respectively.

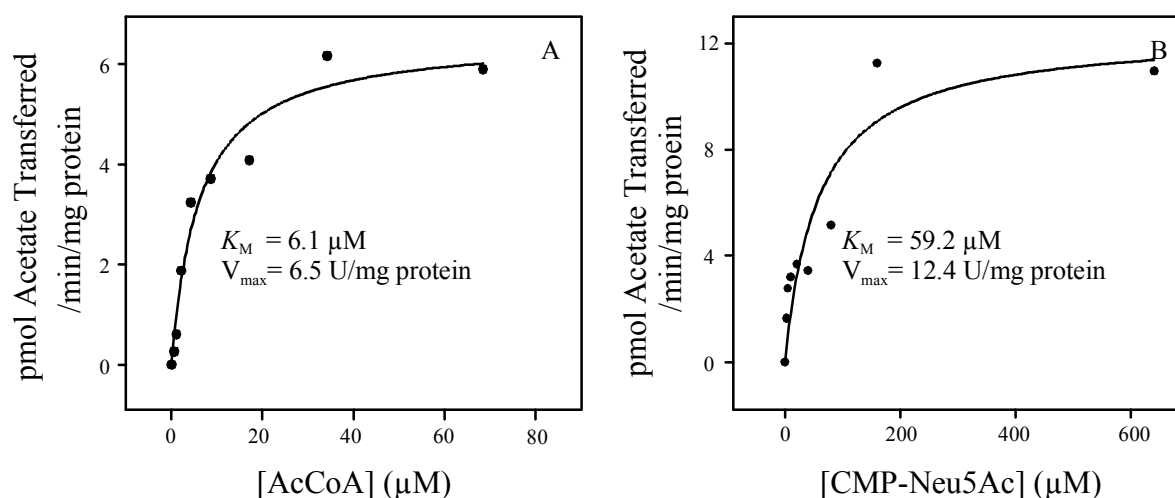


Figure 3-18 Dependence of Sialate-7(9)-OAT Activity on Substrate Concentrations. Fifty μg of microsomal membrane protein was incubated at varying concentrations of AcCoA (A) and CMP-Neu5Ac (B) under optimal conditions (37 $^{\circ}\text{C}$, 15 min, PK buffer containing 50 mM KCl). The concentration of CMP-Neu5Ac in panel A was 600 μM and the concentration of AcCoA in panel B was 8.3 μM . K_M values were determined according to the method of Lineweaver-Burk.

3.2.10 Influence of CoA on 7(9)-OAT Activity

CoA was found to be able to inhibit previously reported OATs [5;6;35;37]. To investigate the influence of CoA on the activity of human colonic 7(9)-OAT, the enzyme assay was carried out under optimal condition (37 °C, 15 min, PK buffer containing 50 mM KCl) in the presence of CoA at different concentrations. Figure 3-19 shows that CoA is a competitive inhibitor of the human colonic 7(9)-OAT, with an apparent K_i for CoA of $11.8 \pm 4.2 \mu\text{M}$ determined by the method of Dixon *et al.* (1958) [114].

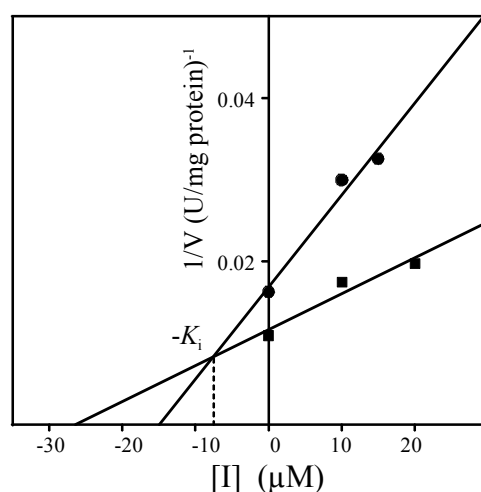


Figure 3-19 Determination of the K_i Value for CoA. Microsomal membrane protein (50 μg) was incubated at concentrations of CoA between 0 and 20 μM together with 8 μM AcCoA (dot) and 16 μM AcCoA (square). 7(9)-OAT activity was determined as described in section 2.2.5.1, page 26. The K_i value was determined as described by Dixon *et al.* (1958) [114].

3.2.11 Effects of Amino Acid-Modifying Reagents on 7(9)-OAT Activity

Previous studies on enzymatic *O*-acetylation of sialic acids in rat liver [36] and bovine submandibular glands showed [35] that amino acids such as cysteine, lysine and tryptophan and histidine were important for full enzyme activity. In order to determine which of those residues is involved in the sialate *O*-acetylation in human colonic Golgi vesicles, the enzyme assays were carried out in the presence of different amino acid-modifying reagents.

As shown in Table 3-2, the activity of 7(9)-OAT decreased considerably in the presence of *p*-CMB, however, DTT has no influence on the 7(9)-OAT activity. These results suggest that at least one free thiol group is essential for the enzymatic *O*-acetylation of sialic acids in human colonic mucosa, while the internal thiol esters and disulfide bridges are not involved in this procedure. Furthermore, the inhibitory effect of DEPC on the enzyme activity suggests

that histidine, tyrosine or lysine may play important roles. At a concentration of 2 mM, the lysine-modifying reagent acid anhydride did not show significant influence on the enzyme activity. On the other hand it inhibited the enzyme activity at the concentration of 10 mM. This is presumably due to the instability of this inhibitor in aqueous solution, thus, higher concentration was required.

Table 3-2 **Effects of Various Amino Acid-Modifying Reagents on colonic 7(9)-OAT Activity.** Golgi vesicles were incubated with CMP-Neu5Ac and [³H]AcCoA in the presence of amino acid-modifiers at the concentration indicated. 7(9)-OAT activity was determined using the standard assay procedure described in section 2.2.5.1, page 26. 7(9)-OAT activity determined in the absence of amino acid-modifiers was set at 100 % (control). The data shown here are expressed as the mean \pm SD of two separated experiments.

Reagents	Target Amino Acid	% of Control (Mean \pm SD)
Control	-	100
2 mM <i>p</i> -CMB	Cys (thiols)	32 \pm 7
2 mM acetic acid anhydride	Lys	90 \pm 5.7
10 mM acetic acid anhydride	Lys	40.7 \pm 17
2 mM DEPC	His, Tyr, Lys	47.4 \pm 14
1 mM DDT	Cys-Cys (-S-S-)	101.4 \pm 11.4

3.3 **The Pathway of Sialate *O*-Acetylation in Human Colonic Mucosa Golgi Vesicles**

3.3.1 **Intactness and Topography of Golgi Vesicles**

To be of physiological significance, it is critical that intact and correctly orientated Golgi vesicles be used for topological studies of 7(9)-OAT. For this purpose, several methods were tried to prepare the high quality Golgi vesicles from human colonic mucosa. The method described by Lepers *et al.* (1979) [21] was adopted but with some modifications so as to

increase the recovery of intact Golgi vesicles. The integrity and orientation of Golgi vesicles was determined in two ways.

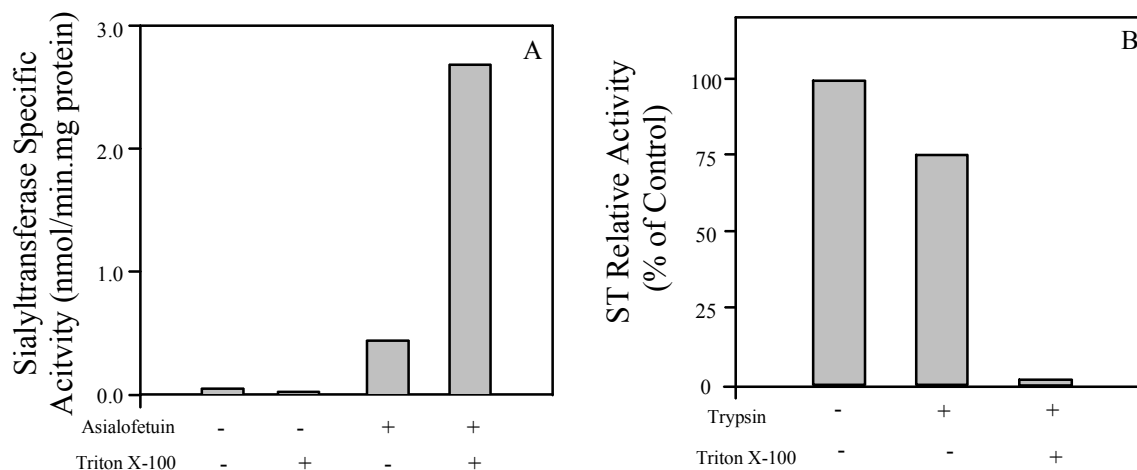


Figure 3-20 Topography and Intactness of Golgi Apparatus-Derived Vesicles. A-ST activity in Golgi vesicles was assayed as described in Alhadeff and Holzinger (1979) [84] in the presence and absence of either Triton X-100 or the exogenous acceptor asialofetuin. B-ST activity of Golgi vesicles prior to and after treatment with trypsin. The trypsin treated Golgi vesicles (protein:trypsin=150:1,w/w) were prepared as described in section 2.2.8, page 31, and the ST activity against asialofetuin subsequently measured.

A quantitative evaluation of the intactness and orientation level of the isolated Golgi vesicles was determined by comparing the ST activity against exogenous substrate in the presence and absence of detergent. As shown in Figure 3-20A, the ST activity against the endogenous substrates was minimal, either in the presence or absence of Triton X-100. However, the ST activity against asialofetuin in the presence of Triton X-100 was 6 times of that detected in the absence of Triton X-100, indicating that about 80 % of the Golgi vesicles were correctly orientated.

Furthermore, Figure 3-20B shows that 25 % of ST activity was lost when Golgi vesicles were pre-treated with trypsin, while the treatment of detergent-disrupted Golgi vesicles with trypsin resulted in the complete abolishment of ST activity. Taken together, these results show that at least 75 % of Golgi vesicles prepared using the procedure described in section 2.2.1.3, page 21, were intact and correctly orientated.

3.3.2 Luminal Orientation of 7(9)-OAT in the Golgi Vesicles

A widely used method for studying the topology of a membrane-associated enzyme is to determine the sensitivity of the enzyme activity to protease digestion [115]. As shown in Figure 3-21, 94 % of the enzyme activity was retained in the Golgi vesicles pre-treated with trypsin. Since at least 75 % of the Golgi vesicles used were intact and correctly orientated, these results suggest that the 7(9)-OAT, at least its active site, is located on the luminal side of isolated Golgi vesicles.

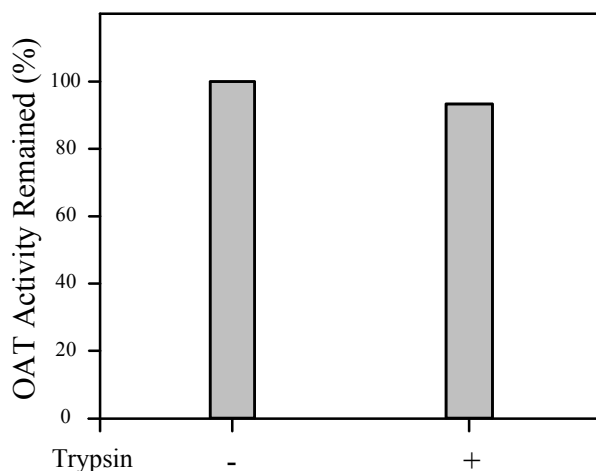


Figure 3-21 Protection of 7(9)-OAT Activity from Trypsin Digestion. Golgi vesicles were treated with trypsin (protein:trypsin=150:1, w/w) at 25 °C for 10 min in 20 mM potassium phosphate buffer, pH 7.0, containing 50 mM KCl and 0.25 M sucrose. The incubation was stopped by the addition of the same ice-cold buffer. After centrifugation at 100 000 x g for 30 min, the Golgi vesicles were surface-washed and used for the determination of 7(9)-OAT activity using the procedure described in section 2.2.5.1, page 26. 7(9)-OAT activity detected in Golgi vesicles prior to trypsin treatment was set at 100 %.

3.3.3 Incorporation of [³H]AcCoA into Luminal CMP-Neu5Ac in the Golgi Vesicles

As shown in the preceding section, the luminal localization of 7(9)-OAT indicates that enzymatic *O*-acetylation of CMP-Neu5Ac occurs in the Golgi lumen. To verify this, CMP-Neu5Ac preloaded Golgi vesicles were incubated with [³H]AcCoA, and the radioactivity incorporated into free sialic acid was analyzed by radio-TLC on silica gel plate.

As shown in Figure 3-22, in comparison to 7(9)-OAT activity detected in non-CMP-Neu5Ac-preloaded Golgi vesicles, a significant increase in the enzyme activity was observed in the Golgi vesicles preloaded with CMP-Neu5Ac for 5 min (Figure 3-22). This suggests that

the enzymatic *O*-acetylation of CMP-Neu5Ac occurs in the Golgi lumen by the luminal localization of 7(9)-OAT. However, extended preloading time did not further increase 7(9)-OAT activity, in fact pre-loading vesicles for 30 min actually led to a decrease in the incorporation of [³H]acetate into CMP-Neu5Ac. The simplest interpretation of this phenomenon is that the activity of 7(9)-OAT is dependent on the concentration of CMP-Neu5Ac in the Golgi lumen.

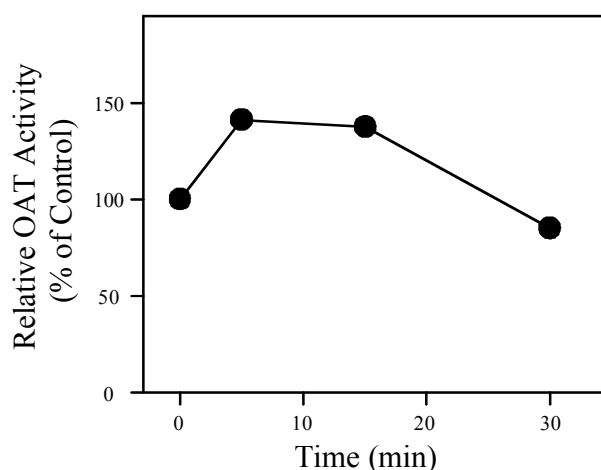


Figure 3-22 Dependence of 7(9)-OAT Activity on the Concentration of Golgi Luminal CMP-Neu5Ac.

Fifty μg of Golgi proteins was preloaded with 500 μM CMP-Neu5Ac in 50 μL of 50 mM Tris-HCl buffer, pH 7.45, containing 10 mM MgCl_2 and CompleteTM protease inhibitor, at 30 °C for time points up to 30 min. After terminating CMP-Neu5Ac transport, Golgi vesicles were pelleted, washed and immediately incubated with [³H]AcCoA under the standard reaction condition outlined in section 2.2.10, page 32. The resulting enzyme products was analyzed, following mild acid hydrolysis and purification, using radio-TLC on cellulose plates (propanol:butanol:0.1M HCl, 2:1:1, v/v/v).

The concentration of CMP-Neu5Ac in the Golgi lumen is regulated by a number of factors. First is the transport of CMP-Neu5Ac from cytosol into the Golgi lumen by a specific CMP-Neu5Ac transporter [23]. It has been reported that the transport of CMP-Neu5Ac occurs rapidly with linear time pointing up to few minutes [19]. Once transported into the Golgi lumen, CMP-Neu5Ac can act as a substrate for STs which transfer Neu5Ac onto glycoconjugates, or alternatively be hydrolyzed [19;22], resulting in the reduction of CMP-Neu5Ac concentration in the Golgi lumen. Taken together, it is highly probable that Golgi vesicles preloaded with CMP-Neu5Ac for 5 min possessed a luminal concentration of CMP-Neu5Ac higher than those preloaded for extended times. Extended preloading times, which

would not further increase the luminal concentration of CMP-Neu5Ac but even possible decreases it, leads to a plateau and then a decrease in the production of CMP-Neu5,9Ac₂.

3.3.4 Involvement of CMP-Neu5Ac Transporter into the Sialate *O*-Acetylation in Human Colonic Mucosa Golgi Vesicles

It has been shown that human colonic 7(9)-OAT catalyzes the *O*-acetylation of CMP-Neu5Ac in the lumen of Golgi vesicles, using AcCoA as the donor substrate. However, both CMP-Neu5Ac and AcCoA are not synthesized in the Golgi vesicles [37;126;127], which suggests that CMP-Neu5Ac and AcCoA must be transported from the cytosol into the Golgi lumen where they can then act as substrates for 7(9)-OAT. Therefore, the influence of various inhibitors of the transport of CMP-Neu5Ac and AcCoA on 7(9)-OAT activity was investigated.

A number of studies show that CMP and CMP-sialic acids derivatives are able to inhibit the translocation of CMP-sialic acids by CMP-Neu5Ac transporter [19;26;128]. Utilizing this knowledge, a two-step procedure was adopted to selectively inhibit the transport of CMP-Neu5Ac. First, Golgi vesicles were preloaded with CMP-Neu5Ac in the presence of various known CMP-Neu5Ac transport inhibitors. After the removal of the inhibitor and non-transported CMP-Neu5Ac, the Golgi vesicles were immediately incubated with [³H]AcCoA. The 7(9)-OAT activity resulting from these incubations was then determined. The structures of inhibitors used are shown in Figure 3-23.

The non-penetrating probe, DIDS, is a widely used inhibitor of the anion transport, in particular of the sugar-nucleotide transport system in the Golgi apparatus [115;129]. The fact that DIDS does not penetrate the membrane, and thus does not affect the anionic-binding enzymes facing the Golgi lumen, provides us the ability to study the influence of CMP-Neu5Ac and AcCoA transport on 7(9)-OAT.

Figure 3-24 shows that DIDS can efficiently inhibit the activity of 7(9)-OAT. This was not the result of the direct inhibitory effect of DIDS on OAT, which has been shown here to be localized in the Golgi lumen. DIDS has been previously reported to be able to inhibit both CMP-Neu5Ac transporter and AcCoA transporter by binding to the basic amino acid residues facing the cytoplasmic side [37;115]. Thereby, the lack of enzyme substrates in the Golgi lumen led to the decrease of 7(9)-OAT activity.

To selectively inhibit the CMP-Neu5Ac transporter, two known specific inhibitors (CMP and KI-8110) [130;131] were used. As shown in Figure 3-24, 7(9)-OAT activity was reduced

in a concentration-dependent manner when Golgi vesicles were preloaded with CMP-Neu5Ac in the presence of CMP. However, no such effect was observed when KI-8110 was used to inhibit CMP-Neu5Ac transport. In fact, at the concentration of 2 mM, no significant influence of KI-8110 on 7(9)-OAT was observed, while 1 mM CMP was capable of reducing the enzyme activity by 50 %. It has been previously shown that 1 mM KI-8110 is not an effective inhibitor of CMP-Neu5Ac transporter as CMP, while only 50 % inhibition being observed even at a concentration of 10 mM [131]. Nevertheless, the observation that KI-8110 could not inhibit the preloading of Golgi vesicles with CMP-Neu5Ac is surprising.

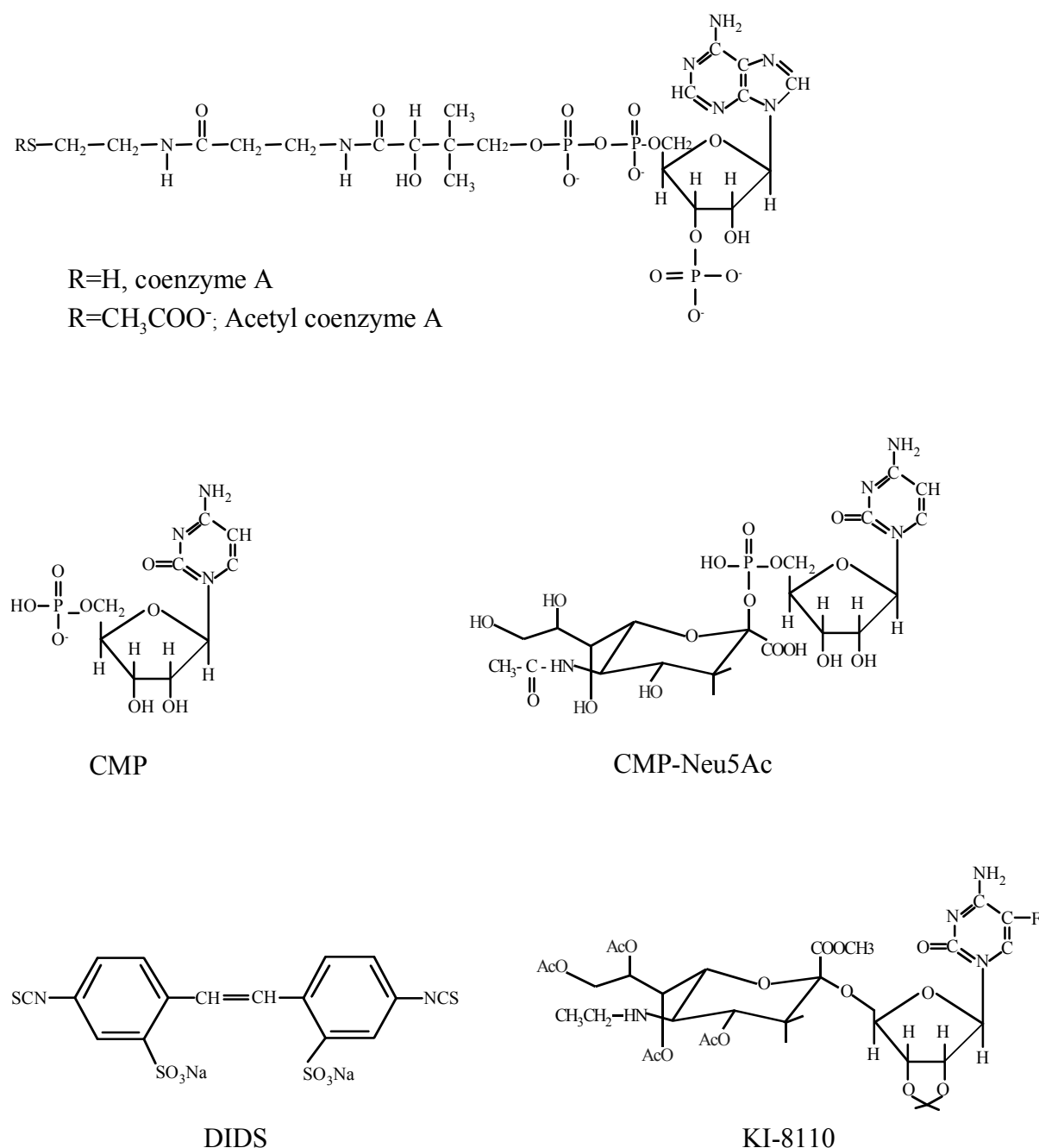


Figure 3-23 Structures of CMP-Neu5Ac and AcCoA Transporter Inhibitors Used in the Experiments.

CoA was also found to be able to reduce the formation of *O*-acetylated CMP-Neu5Ac. It has been previously observed that CoA could be transported into the Golgi lumen [37], in this case a luminal CoA could inhibit the 7(9)-OAT. It is also possible that CoA could inhibit the transport of CMP-Neu5Ac, since a number of other nucleotides, including AMP, are known to be able to inhibit this process [19;132]. A combination of the two effects can also not be ruled out.

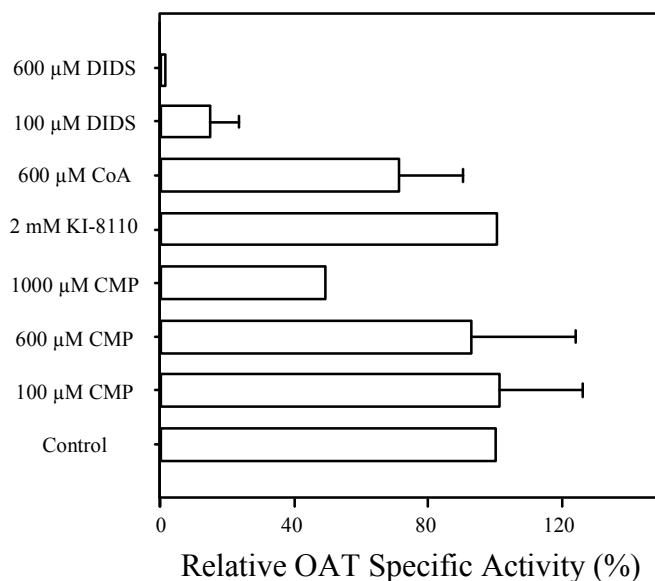


Figure 3-24 Involvement of CMP-Neu5Ac Transporter into Sialate *O*-Acetylation in Human Colonic Mucosa Golgi Vesicles. The incubations of Golgi vesicles with CMP-Neu5Ac (500 μM) were performed in the absence (control) or presence of CMP, DIDS, CoA, KI-8110 under the conditions described in section 2.2.9, page 31. After washing and centrifugation steps, the Golgi vesicles were immediately incubated with [³H]AcCoA. The resulting enzyme products were analyzed, following acid hydrolysis, using radio-TLC on silica gel plates (methanol:chloroform:20 mM CaCl₂, 5:4:1, v/v/v). The radioactive incorporation detected in the control was set at 100 %. The bars show the SD values of two experiments.

3.3.5 Involvement of AcCoA Transporter in Sialate *O*-Acetylation in Human Colonic Mucosa Golgi Vesicles

In order to study the importance of the AcCoA transport on sialate *O*-acetylation in human colonic mucosa, CMP-Neu5Ac-preloaded Golgi vesicles were incubated with [³H]AcCoA in the presence and absence of CoA, DIDS and CMP, and the activity of 7(9)-OAT was determined. The enzyme activity detected in the absence of AcCoA transporter inhibitors was set at 100 %.

CoA is a competitive inhibitor of the putative AcCoA transporter [5;35;37], while DIDS inhibits AcCoA transport in a similar fashion to how it inhibits other transport processes [37]. Because of the procedure used, the transport of CMP-Neu5Ac into the Golgi lumen occurred prior to the addition of CoA and DIDS, that is, only the transport of AcCoA can be influenced. Therefore, as shown in Figure 3-25, the depletion of the AcCoA pool in the Golgi lumen by the addition of AcCoA transporter inhibitors resulted in a decrease in the *O*-acetylation of CMP-Neu5Ac. Moreover, the inhibitory effect of CoA was concentration-dependent, which is in agreement with the fact that CoA is a competitive inhibitor of the AcCoA transporter, and strongly suggests that sialate *O*-acetylation in the colonic Golgi vesicles is regulated by the transport of AcCoA.

Moreover, as shown in Figure 3-25, CMP was found to decrease the 7(9)-OAT activity in a concentration-dependent way. This was not the result of the inhibitory effect of CMP on CMP-Neu5Ac transport, since CMP was added after the preloading of Golgi vesicles with CMP-Neu5Ac. However, it has been reported that CMP could be transported into Golgi lumen [133], and thus could inhibit the 7(9)-OAT. Another possibility is that CMP could inhibit the transport of AcCoA, since both CMP and CoA have a nucleoside moiety. It is also due to the combination of the two effects.

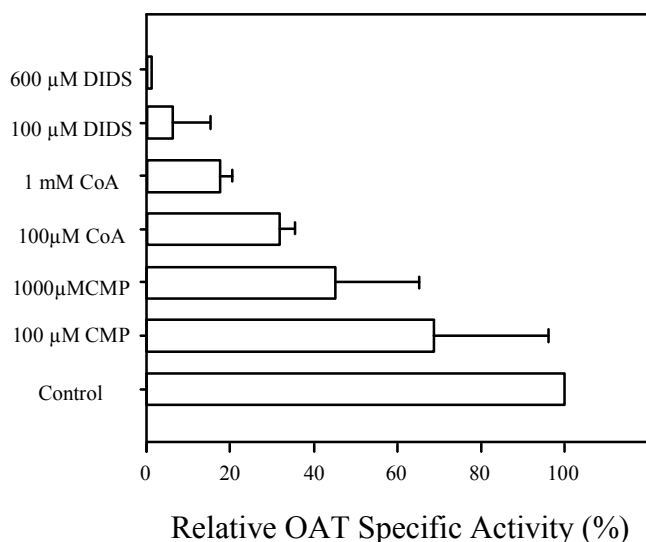


Figure 3-25 Participation of the AcCoA Transporter in the Sialate *O*-Acetylation in Human Colonic Mucosa Golgi Vesicles. CMP-Neu5Ac-preloaded Golgi vesicles were incubated with [^3H]AcCoA in the absence (control) and presence of the substances indicated above. The resulting enzyme products were hydrolyzed and analyzed by radio-TLC on silica gel plates, which were developed in methanol:chloroform:20 mM CaCl_2 (5:4:1, v/v/v). The radioactive incorporation into sialic acid detected in the control was set at 100 %. The bars show the SD values of two experiments.

3.3.6 Transferring of Neu5,9Ac₂ from CMP-Neu5,9Ac₂ to Endogenous Glycoconjugates

It has been shown in the previous sections that in human colonic Golgi vesicles sialate *O*-acetylation occurs at the sugar-nucleotide level and is catalyzed by a specific 7(9)-OAT. However, in nature, sialic acids are typically found at the terminal position of glycans present on glycoproteins and glycolipids [2]. Therefore, investigations were carried out to determine if newly synthesized CMP-linked *O*-acetylated sialic acid could be transferred by endogenous STs to glycoprotein and glycolipid acceptors in the same Golgi vesicles.

Following the incubation of Golgi vesicles with CMP-Neu5Ac and [³H]AcCoA, DIDS was added to terminate transporter activities. The mixtures were then further incubated for the times indicated in Figure 3-26. Due to the ability of DIDS to inhibit the transport of CMP-Neu5Ac and AcCoA, further synthesis of *O*-acetylated CMP-Neu5Ac could be effectively halted without affecting other enzymatic processes, such as the sialyltransferase activity in the Golgi lumen. In this way the final destination of *O*-acetylated sialic acid could be monitored. The incorporation of radioactivity associated with free CMP-sialic acid in the acid-soluble fraction and glycoconjugate-bound sialic acid in the acid-insoluble fraction was determined by radio-TLC on silica gel plates.

As shown in Figure 3-26, an increase in the total and acid-soluble radioactivity was observed when Golgi vesicles were incubated for a further 1 h after the addition of DIDS. This indicates that CMP-Neu5Ac and AcCoA in the Golgi lumen were still being utilized as substrates by the 7(9)-OAT for as long as 1 h following the termination of the corresponding transport activities. However, following a further 2 h' incubation post-DIDS addition, no further neo-CMP-[³H]Neu5,9Ac₂ was synthesized in the Golgi vesicles (Figure 3-26). This suggests that DIDS completely blocked the transport of AcCoA and CMP-Neu5Ac.

The incorporation of [³H]acetate into the acid-insoluble fraction, on the other hand, increased linearly from 1 hour post-DIDS addition to a maximum at 4 h. A corresponding decrease in the radioactivity associated with free CMP-sialic acid was also observed at time points over 2 h. This indicates that neo-synthesized CMP-Neu5,9Ac₂ was transferred to endogenous acid-insoluble glycoconjugate-bound sialic acids by a ST associated with human colonic Golgi vesicles. It should be noted that ST activities measured against endogenous substrate *in vitro* are generally very low, as was also seen in Figure 3-20A (section 3.3.1, page 59). The transfer of approximately 14 % of [³H]Neu5,9Ac₂ from newly synthesized CMP-[³H]Neu5,9Ac₂ into endogenous glycoconjugates is, therefore, significant, and implies

that a ST having donor substrate preference for CMP-Neu5,9Ac₂ exists in human colonic Golgi vesicles.

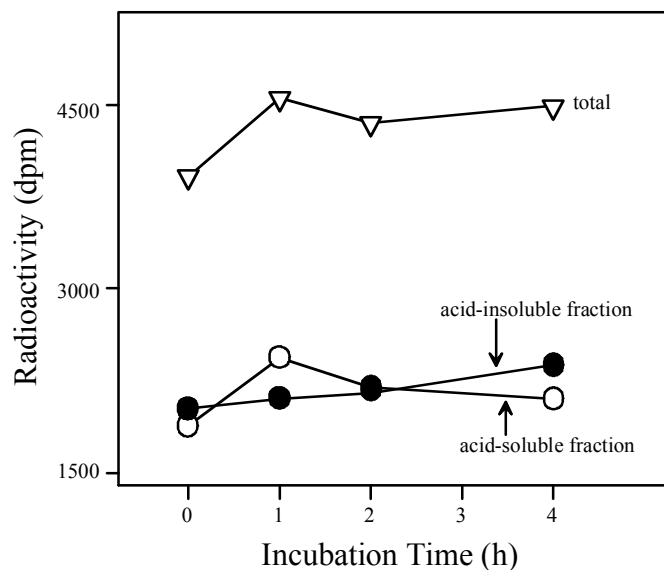


Figure 3-26 The Transfer of Neu5,9Ac₂ from Neo-Synthesized CMP-Neu5,9Ac₂ to Endogenous Glycoconjugates in the Golgi Vesicles. Following the incubation of Golgi vesicles (100 μ g) with 200 μ M CMP-Neu5Ac and [³H]AcCoA (0.25 μ Ci, 0.3 μ M) in 50 mM PK buffer, pH 7.0, at 37 °C for 40 min, DIDS was added to a final concentration of 1 mM. The mixtures were then further incubated at 37 °C for 1, 2 and 4 hours, following which the reactions were terminated by the addition of propionic acid. The radioactivity associated with the acid-soluble and acid-insoluble fractions were analyzed by radio-TLC on silica gel plates (methanol:chloroform:20 mM CaCl₂, 5:4:1, v/v/v).

3.3.7 Incorporation Rate of Neu5Ac to Neu5,9Ac₂ onto Various Glycoproteins

Generally, each sialic acid linkage is generated by a single or more sialyltransferases which show the specificity not only for the linkage but also for the acceptor [2;29]. Therefore, the direct transfer of Neu5,9Ac₂ from CMP-Neu5,9Ac₂ to endogenous glycoconjugates should depend not only on the synthesis of CMP-Neu5,9Ac₂, but also on the acceptor substrate specificities of the sialyltransferases involved. To further explore this, the incorporation rate of Neu5,9Ac₂ into different potential acceptors was compared with that of Neu5Ac by employing human colonic Golgi STs and rat liver α 2,6-ST (EC.2.4.99.1).

As shown in Figure 3-27, using asialofetuin as the acceptor and CMP-Neu5,9Ac₂ as the donor, a different substrate specificity for the two STs used was observed. Rat liver α 2,6-ST showed the lower transfer rate of Neu5,9Ac₂ compared with that of Neu5Ac, which was reduced to 66 %. This is in good agreement with the previous reports [65;67], and indicates

that rat liver $\alpha 2,6$ -ST is not specific for CMP-Neu5,9Ac₂. In contrast, the human colonic Golgi STs transferred Neu5,9Ac₂ to asialofetuin at a rate 5-fold of that observed for Neu5Ac. These results suggest that there might exist one colonic ST preferring to utilize CMP-Neu5,9Ac₂ as the donor substrate.

On the other hand, when asialo-BSM was used as the acceptor substrate, the incorporation rate of Neu5,9Ac₂ to asialo-BSM was decreased when compared with that of Neu5Ac, suggesting that asialo-BSM was not the optimal acceptor substrate for Neu5,9Ac₂.

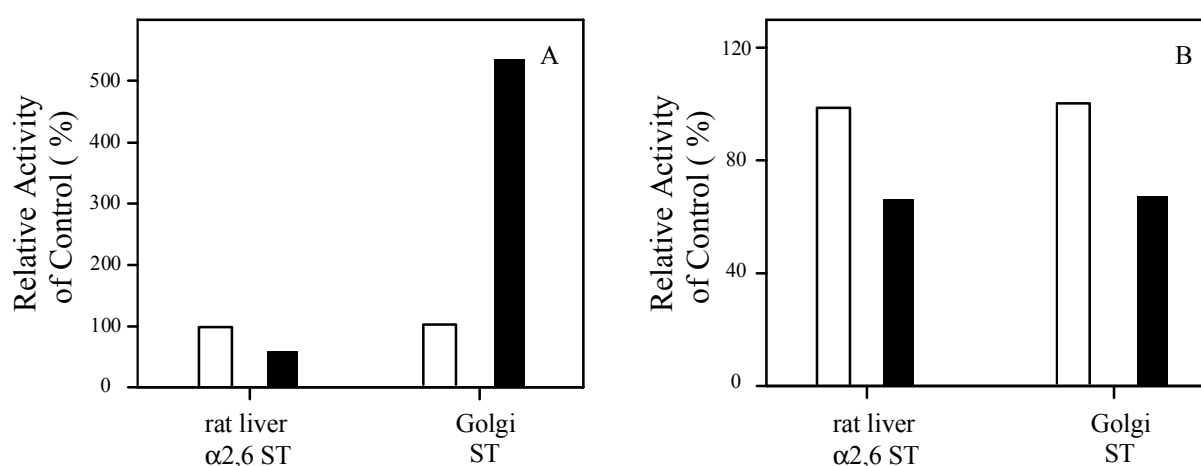


Figure 3-27 The Incorporation Rate of Neu5,9Ac₂ into Asialofetuin or Asialo-BSM by Human Colonic Mucosa STs and Rat liver $\alpha 2,6$ -ST. Using CMP-[³H]Neu5,9Ac₂ (black bar) as the donor substrate, the ST activity against asialofetuin (A) and asialo-BSM (B) was determined, respectively. Control was performed using CMP-[¹⁴C]Neu5Ac (white bar), and the ST activity detected in the control was set at 100 %.

3.4 Solubilization of Membrane-Bound Sialate-7(9)-O-Acetyltransferase

3.4.1 Pre-Extraction Microsomal Proteins with EDTA, 1 M KCl and Freeze-Thawing

The procedure to remove water-soluble and peripheral membrane proteins is called pre-extraction [134]. Since peripheral proteins are bound to membranes primarily by electrostatic and hydrogen-bond interactions, therefore, they usually could be released from the membrane by changing the pH or ionic strength, or by using chelating agents [135]. Water-soluble

proteins could be removed by disrupting membranes without detergent, following the removal of membranes by ultracentrifugation [134].

As shown in Figure 3-28, the treatment of microsomes with EDTA, either at 1 mM or 100 μ M concentration, resulted in a 50 % decrease in enzyme activity of the membrane pellet. However, no enzyme activity was observed in the EDTA extraction supernatant. Moreover, two cycles of freeze-thawing or 1 M KCl extraction did not release the 7(9)-OAT in the supernatant, because 80 % of the enzyme activity remained in the membrane pellet treated in such ways (Figure 3-28). These results suggest that 7(9)-OAT cannot be extracted from the membrane by pre-extraction.

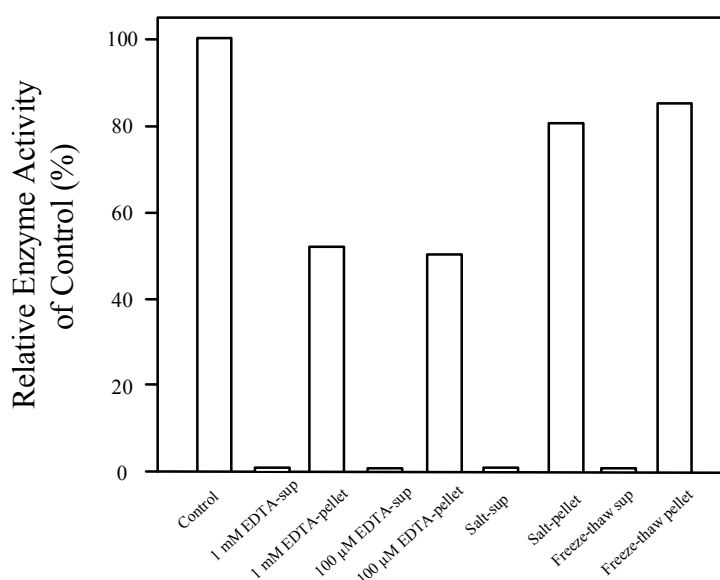


Figure 3-28 Extraction of the 7(9)-OAT Activity Using EDTA, 1 M KCl and Freeze-Thawing. Two hundred microgram of microsomal membranes was extracted with EDTA, washed with 1 M KCl, and put through 2 freeze-thaw cycles, respectively. After centrifugation at 100 000 x g for 30 min, the resulting supernatants (sup) and pellets were used to determine 7(9)-OAT activity. The enzyme activity detected in the membrane fractions prior to these treatments was set at 100 % (control).

3.4.2 Solubilization and Stabilization of 7(9)-OAT

Various detergents including nonionic detergents (Triton X-100, Nonidet P40, LUBROL Px and octylglucoside) and zwitterionic detergents (Zwittergent 3-12 and CHAPS) were examined for their abilities to solubilize 7(9)-OAT in an active form. The structures of those detergents are shown in Appendix II. For each detergent, the solubilization was performed at different protein to detergent ratios varying from 10:1 to 1:10. The solubilization efficiency is

estimated by quantitative analysis of the 7(9)-OAT activity and protein amount before ultracentrifugation and in the supernatant after ultracentrifugation.

Of all the detergents tested, CHAPS (0.9 % w/v) was found to be able to solubilize 7(9)-OAT, however, only a small amount of activity was detected in the soluble form. As shown in Figure 3-29A, the incubation of [³H]AcCoA and AcCoA with CHAPS-solubilized microsomal membrane proteins resulted in a radioactive peak, co-migrating with authentic Neu5,9Ac₂ on silica gel plates. However, only about 1 % of the original membrane-bound enzyme activity (Figure 3-29B) was recovered in the soluble form.

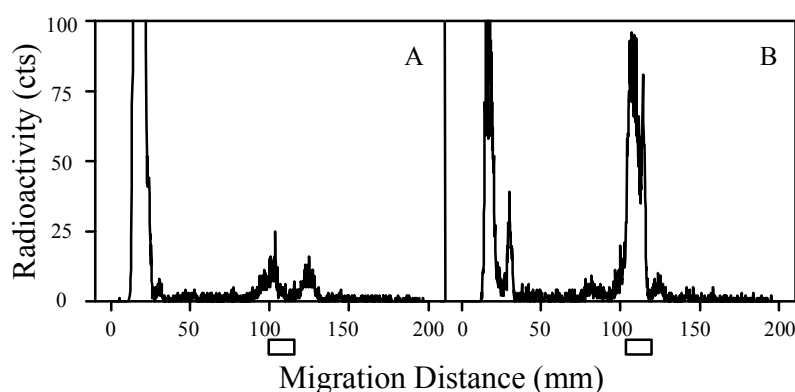


Figure 3-29 Solubilization of 7(9)-OAT from Human Colonic Microsomes with CHAPS. Microsomal proteins were solubilized with 0.9 % CHAPS using the procedure described in section 2.2.13.4, page 34. After centrifugation at 100 000 x g for 30 min, the supernatant (A) was used for the determination of 7(9)-OAT activity against CMP-Neu5Ac. The enzyme activity in the microsomes prior to the addition of CHAPS (B) was also determined. The enzyme products were hydrolyzed and analyzed with radio-TLC on silica gel plates (methanol:chloroform:20mM CaCl₂, 5:4:1, v/v/v). The position of BIAL reagent-stained authentic Neu5,9Ac₂ is indicated.

To stabilize 7(9)-OAT, DTT and glycerol were added during the solubilization of microsomal proteins. The enzyme activity detected in the microsomes prior to the addition of CHAPS was set at 100 %. As shown in Figure 3-30, only about 1 % of the membrane 7(9)-OAT activity was recovered in the soluble form, and the presence of DTT or glycerol did not increase this value.

Moreover, the binding of enzyme to substrate is thought to be able to increase the protein stability. In some cases, this stabilizing effect has been valuable for membrane protein isolation [136]. However, the presence of CMP-Neu5Ac during the solubilization of microsomal proteins provided no stabilizing effect on the solubilized 7(9)-OAT (Figure 3-30).

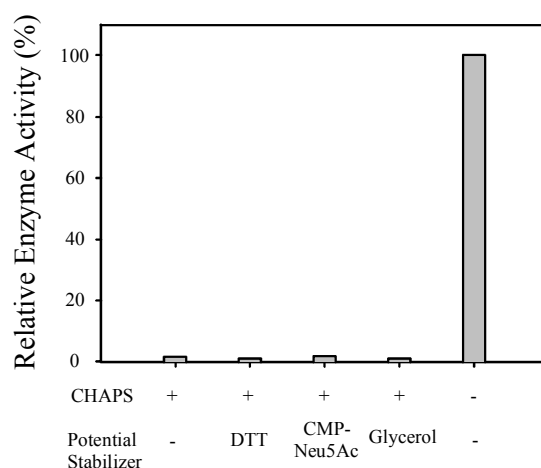


Figure 3-30 Solubilization of Membrane 7(9)-OAT in the Presence of DTT, Glycerol and CMP-Neu5Ac. Microsomal proteins were solubilized with 0.9 % (w/v) CHAPS in the presence of 1 mM DTT, 300 μ M CMP-Neu5Ac and 20 % (v/v) glycerol, respectively, as described in section 2.2.13.4, page 34. After centrifugation at 100 000 \times g for 30 min, the resulting supernatant was used to determine 7(9)-OAT activity against CMP-Neu5Ac. The enzyme activity in the microsomes prior to the addition of CHAPS was set at 100 %.

A number of membrane-associated enzymes have been successfully reactivated by the addition of lipids following solubilization [137;138]. Hence, lecithin was added to the solution of solubilized microsomal proteins, and the activity of 7(9)-OAT was measured. Unfortunately, as shown in Figure 3-31, no reactivation of the solubilized 7(9)-OAT was achieved in the presence of lecithin.

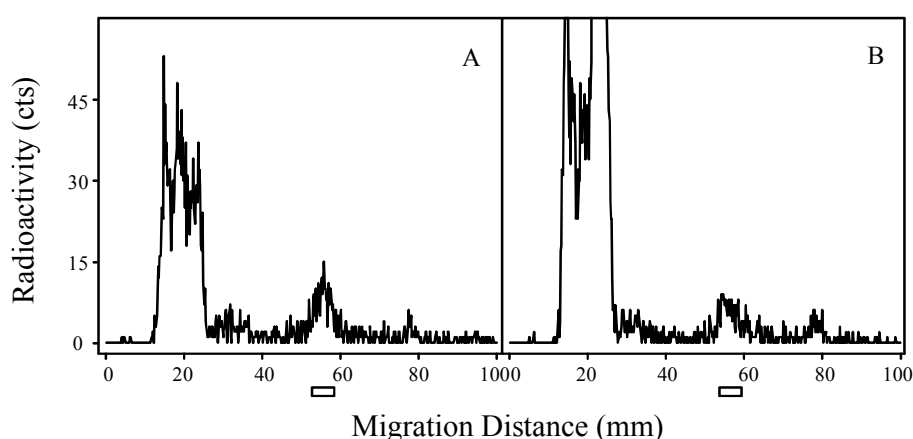


Figure 3-31 Effect of Lecithin on the Activity of Soluble 7(9)-OAT. Microsomal protein was solubilized with CHAPS, and used to determine 7(9)-OAT activity against CMP-Neu5Ac, in the absence (A) and presence (B) of lecithin-saturated suspension. The resulting enzyme products were hydrolyzed, purified, and analyzed by radio-TLC on silica gel plates (methanol:chloroform:20 mM CaCl_2 , 5:4:1, v/v/v). The migration distance of BIAL reagent-stained authentic Neu5,9Ac₂ is indicated.

3.4.3 Pitfall in the Identification of Radiolabeled Products Resulting from the Incubation of Solubilized Microsomal Proteins with [^3H]AcCoA

During the course of trying to solubilize the 7(9)-OAT from microsomes, it was found that special attention should be paid when radio-TLC analysis is performed on a cellulose plate, which is commonly used for the identification of *O*-acetylated sialic acids. Following the incubation of solubilized microsomal proteins with [^3H]AcCoA and CMP-Neu5Ac, the resulting enzyme products were hydrolyzed, purified and analyzed with radio-TLC on cellulose and silica gel, respectively.

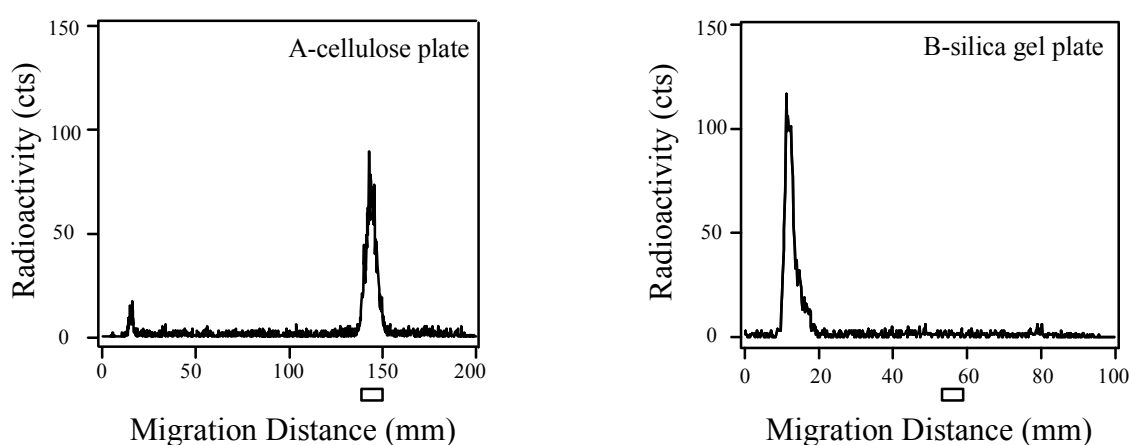


Figure 3-32 Radio-TLC Analyses of the Purified Products Resulting from the Incubation of Solubilized Microsomal Proteins with [^3H]AcCoA and CMP-Neu5Ac. Following the incubation of solubilized microsomal proteins with [^3H]AcCoA and CMP-Neu5Ac, the products were hydrolyzed, purified as described in section 2.2.5.2, page 28, and analyzed with radio-TLC on a silica gel 60 plate (10x10 cm, methanol: chloroform: 20 mM CaCl_2 , 5:4:1, v/v/v) and a cellulose plate (20x20cm, propanol: butanol: 0.1 M HCl, 2:1:1, v/v/v), respectively. The migration position of BIAL reagent-stained authentic Neu5,9Ac₂ is indicated.

As shown in Figure 3-32, different chromatographic profiles were obtained, even though aliquots from the same sample were analyzed. A radioactive peak co-migrating with authentic Neu5,9Ac₂ was observed on the cellulose plate (Figure 3-32A), which is sensitive to alkaline treatment. However, on the silica gel plate the only radioactive peak observed was at the point of origin (Figure 3-32B). These results indicated that the assumed [^3H]Neu5,9Ac₂ peak on cellulose is not *O*-acetylated sialic acid. This was further confirmed since this product was resistant to the action of influenza C virus esterase and sialate-pyruvate-lyase. Therefore, to identify *O*-acetylated sialic acids, it is essential to utilize at least two TLC systems in

combination with specific methods, such as the treatment with influenza C virus esterase and sialate-pyruvate-lyase.

Our findings and the reports of the other groups [4;7;70] showed that the activity of 7(9)-OAT is extremely sensitive to detergent, and the enzyme activity is strictly dependent on the membrane integrity [4;70]. The loss of enzyme activity following solubilizing proteins from membranes could be due to a variety of reasons, including the loss of associated cofactors or certain subunits.

3.5 Sialate-*O*-Acetyltransferase (OAE): Sialate De-*O*-Acetylation in Human Colonic Mucosa

3.5.1 Distribution of OAE in Human Colonic Mucosa

Subcellular fractions (cytosol, lysosomes and microsomes) were simultaneously prepared from human colonic homogenates. The β -galactosidase activity was used as a marker for the lysosomal fraction, while sialyltransferase activity was for the microsomal fraction. Table 3-5 outlines a typical result for the distribution of these marker enzymes in the various fractions obtained from the uninvolved colonic mucosa following differential centrifugation.

Table 3-5 The Distribution of Marker Enzyme Activities in Various Subcellular Fractions Isolated by Differential Centrifugation. 1U of galactosidase=1 nmol of MU produced/min.; 1U of ST=1 nmol of Neu5Ac transferred /h.

Enzyme Specific Activity (U/mg protein)	Post 3 000 x g Pellet	Lysosome	Cytosol	Microsome
β -galactosidase	3.95	13.4	0	1.2
Sialyltransferase	Not detected	Not detected	0	3.6

The expression of *O*-acetylated sialic acids in human colonic mucosa indicates that the OAEs should be present in the same tissue. To verify this, the OAE activity in subcellular fractions was assayed by using Neu5,9Ac₂, Neu5,7(8),9Ac₃ or BSM as the substrate,

respectively. Here we refer to the esterase activity against Neu5,9Ac₂ as 9-OAE, while that against Neu5,7(8),9Ac₃ as oligo-OAE.

Using BSM as exogenous substrate, quite low esterase activity was observed in the cytosolic fraction and the membrane fraction. One explanation for this is that, *in vitro*, the sticky property of BSM hindered the action of esterase. Therefore, we only concentrated on the esterase against free *O*-acetylated sialic acids.

As shown in Figure 3-34, the esterase activity against Neu5,9Ac₂ or Neu5,7(8),9Ac₃ has been detected not only in the cytosol but also in the microsomes and lysosomes. However, the OAE activity detected in the microsomes was quite low. This is probably the result of residual lysosomes being present in the microsomal fraction, as is shown by the presence of β -galactosidase activity in the isolated microsomal fraction (Table 3-5). Therefore, these results suggest that at least two forms of OAE exist in human colonic mucosa, with one being localized in the cytosol, and the other being membrane-associated, most probably in the lysosomes.

Moreover, the distribution of esterase activity against MU-Ac in different subcellular fractions was found to be similar with that of OAE, however, higher enzyme activity was detected in the microsomal fraction.

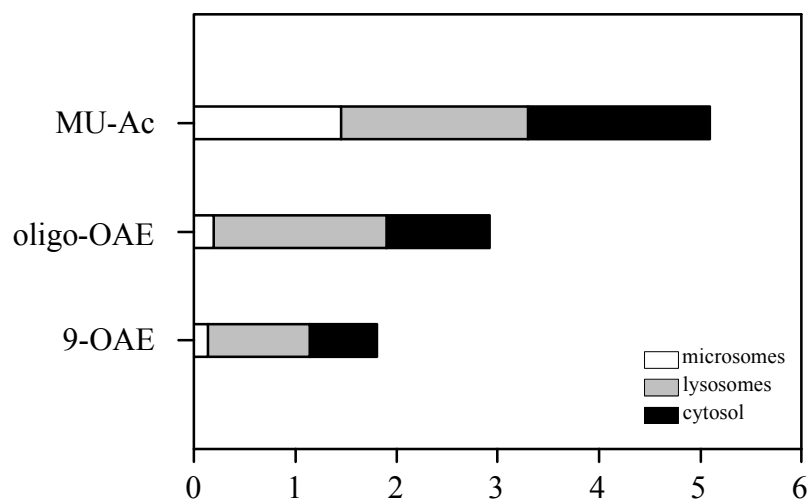


Figure 3-34 Subcellular Distribution of OAE Activities in Human Colonic Mucosa. The esterase specific activity was measured by incubating subcellular fractions with Neu5,9Ac₂ and Neu5,7(8),9Ac₃, respectively, as described in section 2.2.14, page 35. One unit of OAE activity equals to 1 nmole of acetic acid released from *O*-acetylated sialic acids per min under the experimental condition used. One unit of esterase activity against MU-Ac equals to 0.1 mmole of MU released from MU-Ac per min under the condition used.

3.5.2 Enzymatic Activity on *O*-Acetyl Esters from Mono- and Di-*O*-Acetylated Sialic Acids Shows Specificity for the Hydrolysis of 9-*O*-Acetyl Groups

To confirm the enzymatic hydrolysis of *O*-acetyl residues from mono-*O*-acetylated sialic acids, the enzyme products were examined by fluorometric HPLC. As shown in Figure 3-35, following the incubation of Neu5,7Ac₂ (peak 2) and Neu5,9Ac₂ (peak 3) with cytosolic proteins, the amount of Neu5,9Ac₂ (peak 3) was decreased with a corresponding increase in the amount of Neu5Ac (peak 1), while no reduction in the amount of Neu5,7Ac₂ was observed after short incubation times. However, no degradation of *O*-acetyl groups from Neu5,9Ac₂ was observed when denatured proteins were used (Figure 3-35B). Therefore, the hydrolysis of mono-*O*-acetylated sialic acids is catalyzed by an enzyme specific for the cleavage of sialate 9-*O*-acetyl ester. The alkaline treatment of Neu5,9Ac₂ resulted in the complete de-*O*-acetylation of Neu5,9Ac₂ (Figure 3-35C), identifying peak 4 as being the reagent peak. The same results were obtained when membrane proteins were used.

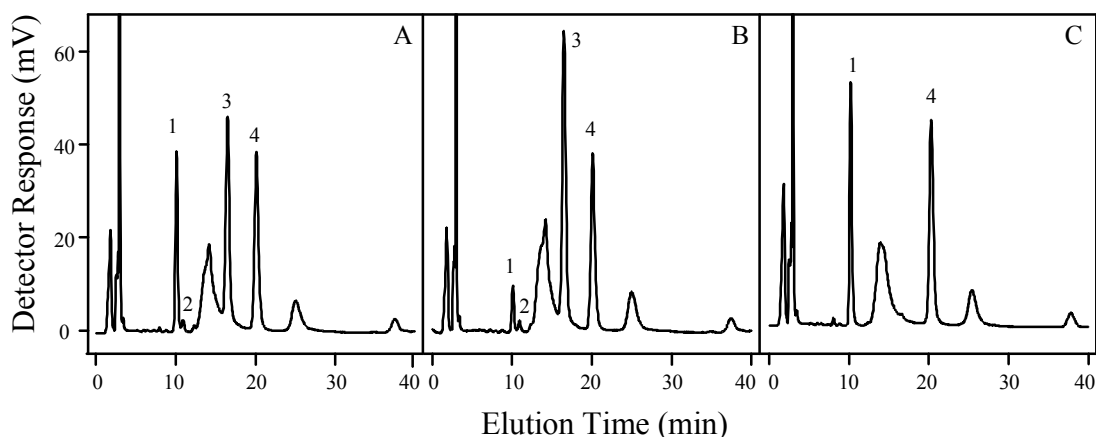


Figure 3-35 Hydrolysis of 9-*O*-Acetyl Groups from Neu5,9Ac₂ by the Cytosolic OAE. Following the incubation of cytosolic proteins (A) or heat-denatured proteins (B) with Neu5,9Ac₂, the enzyme products were examined by fluorometric HPLC. The saponification of Neu5,9Ac₂ was fulfilled with 5 % NH₃ at 37 °C for 1 h (C). Peak 1, Neu5Ac; Peak 2, Neu5,7Ac₂; Peak 3, Neu5,9Ac₂; Peak 4, reagent peak.

The hydrolysis of Neu5,7(8),9Ac₃, a mixture of Neu5,7,9Ac₃ and Neu5,8,9Ac₃, was also studied. Figure 3-36 shows that the non-specific de-*O*-acetylation of Neu5,7(8),9Ac₃ induced by alkaline treatment resulted in the formation of Neu5Ac. However, following the incubation of this mixture with cytosolic proteins, the reduction in the amount of Neu5,7(8),9Ac₃ was accompanied with an increase in the level of not only Neu5Ac, but also Neu5,7Ac₂ and Neu5,9Ac₂ (Figure 3-36 and 3-37). This suggests that the enzymatic hydrolysis of

Neu5,7,9Ac₃ occurred first by cleaving the 9-*O*-acetyl group, which led to the formation of Neu5,7Ac₂.

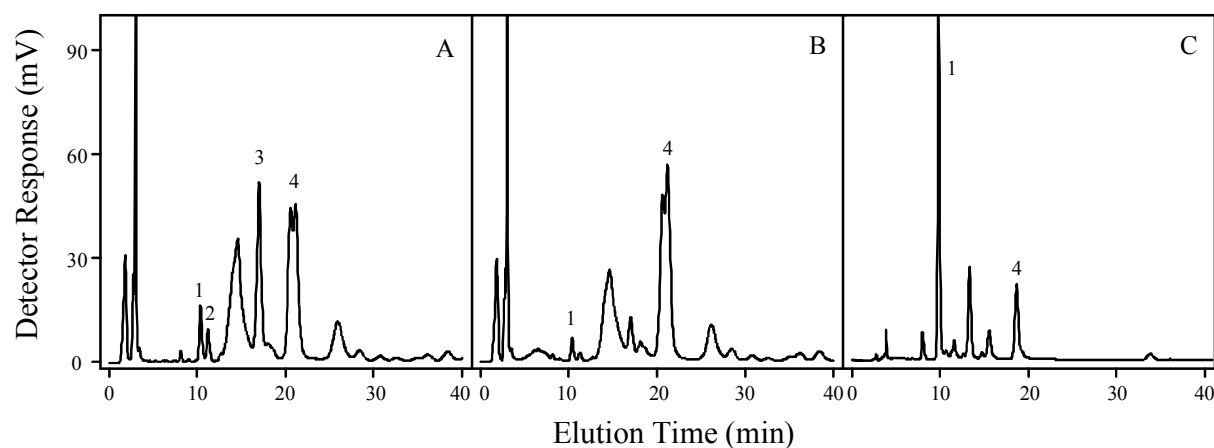


Figure 3-36 Hydrolysis of *O*-Acetyl Groups from Neu5,7(8),9Ac₃ by the Cytosolic OAE. Cytosolic protein (A) or heat-denatured protein (B) was incubated with 2.5 mM Neu5,7(8),9Ac₃, and the enzyme products were analyzed by fluorometric HPLC. The saponification of Neu5,7(8),9Ac₃ was fulfilled with 5 % NH₃ at 37 °C for 1 h (C). Peak 1, Neu5Ac; Peak 2, Neu5,7Ac₂; Peak 3, Neu5,9Ac₂; Peak 4, Neu5,7(8),9Ac₃/reagent peak.

After 1 h, a drop of Neu5,7Ac₂ level and a corresponding increase in Neu5Ac was observed (Figure 3-36). Since Neu5,7Ac₂ is a poor substrate for the cytosolic OAE as shown in Figure 3-35, the drop in the level of Neu5,7Ac₂ amount observed here was not due to the action of cytosolic OAE. On the other hand, it was previously reported that the migration half-life of free Neu5,7Ac₂ is 4-8 h at physiological conditions (37 °C, pH 7.0) [9]. Therefore, it is likely that the spontaneous migration of acetyl group from 7- to 9-position under the condition used for esterase assay (37 °C, pH 7.45) resulted in the reduction in the amount of Neu5,7Ac₂. The resulting Neu5,9Ac₂ then can be further hydrolyzed by the cytosolic OAE.

Under the condition (37°C, pH 7.45) used for esterase assay, the rate of spontaneous migration of the acetyl group from 7- to the 9-position of sialic acid is slow. The possibility of the involvement of a migrase, which has been postulated to be able to increase the migration rate at least 100-fold, is low because this activity has been found to be localized in microsomal fraction [35]. Therefore, the continuously rising in the amount of Neu5,9Ac₂ (Figure 3-37) was not likely to be the result of the isomerization of Neu5,7Ac₂, which was produced by the enzymatic hydrolysis of Neu5,7,9Ac₃.

In contrast, it was reported that the migration of *O*-acetyl groups from 8- to 9-position may occur so fast that Neu5,8Ac₂ cannot be isolated in a stable state [9]. Thus, it is proposed that the enzymatic de-*O*-acetylation of Neu5,8,9Ac₃ occurs first at the position of 9-*O*-acetyl group, and the acetyl group of the resulting Neu5,8Ac₂ undergoes a very rapid migration to the 9-position, which leads to an increase in the amount of Neu5,9Ac₂. The resulting Neu5,9Ac₂ could then be hydrolyzed by the cytosolic OAE with a correspondingly increase in the amount of Neu5Ac (Figure 3-37).

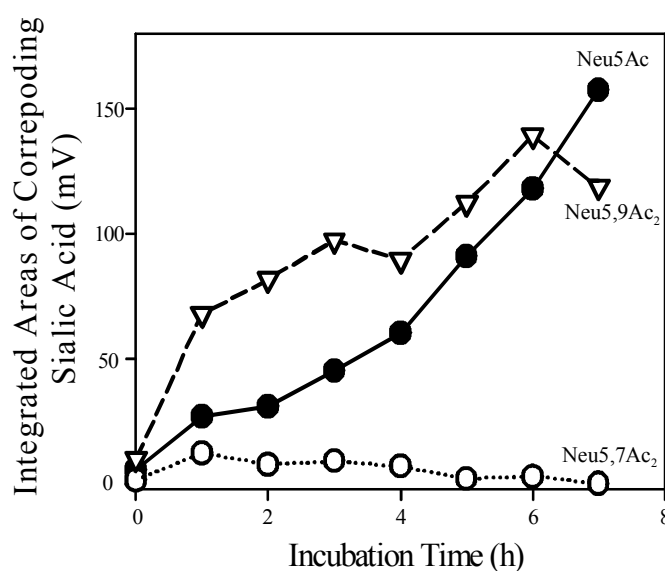


Figure 3-37 Positional Specificity of the Cytosolic OAE on *O*-Acetyl Esters from Neu5,7(8),9Ac₃. Following the incubation of Neu5,7(8),9Ac₃ with cytosolic proteins, the enzyme products were analyzed by fluorometric HPLC at various times. Control was performed using heat-denatured proteins. The quantity of sialic acid was determined from the area under the individual sialic acid peak obtained following HPLC.

3.6 Comparison of the Level of *O*-Acetylated Sialic Acids and the Activities of 7(9)-OAT and OAE in the Uninvolved and Cancerous Colonic Mucosa

3.6.1 A Decrease in the Expression Level of *O*-Acetylated Sialic Acids in the Cancerous Mucosa

The nature of sialic acids present in the cytosolic and membrane fractions prepared from cancerous mucosa was also analyzed by fluorometric HPLC and GC-MS (Table 3-7). The presence of glycoconjugate-bound Neu5Ac and Neu5,9Ac₂ in the cytosolic and membrane

fractions was confirmed. However, no oligo-*O*-acetylated sialic acids and Neu5Gc as well as free *O*-acetylated sialic acids were detected by GC-MS analysis.

Table 3-7 The Nature of Sialic Acids Present in the Subcellular Fractions from Cancerous Mucosa.

Sialic acids were purified from the cytosolic fraction or microsomal fraction, and were analyzed by fluorometric HPLC and GC-MS as described in section 2.2.4, page 24.

Subcellular fractions	Fluorometric HPLC	GC-MS
Cytosol	Neu5Ac	Neu5Ac
	Neu5,9Ac ₂	Neu5,9Ac ₂
	Oligo- <i>O</i> -acetylated Neu5Ac (?)	
Microsomes	Neu5Ac	Neu5Ac
	Neu5,9Ac ₂	Neu5,9Ac ₂
	Oligo- <i>O</i> -acetylated Neu5Ac (?)	
Free sialic acids in cytosol	Neu5Ac	Neu5Ac
	Neu5,9Ac ₂	
	Oligo- <i>O</i> -acetylated Neu5Ac (?)	

Using fluorometric HPLC analysis, the relative expression level of *O*-acetylated sialic acids in the uninvolved and cancerous mucosa from the same patient was determined (Table 3-6). The level of mono- and oligo-*O*-acetylated sialic acids in the uninvolved mucosa was $22.4 \pm 10.8\%$ (n=13) and $5.0 \pm 4.6\%$ (n=13), respectively. These results are compatible with the previous reports [94;95]. When compared with the uninvolved mucosa, the cancerous mucosa expressed very significantly lower levels of the mono- (t-paired test, $p < 0.005$) and oligo-*O*-acetylated sialic acids (t-paired test, $p < 0.005$), however, the higher level of Neu5Ac (t-paired test, $p < 0.0001$). This is supposed to be a result of a decrease in the amount of *O*-acetylated sialic acids.

Table 3-6 Sialic Acids from the Uninvolved and Cancerous Colonic Mucosa.

Tissue Nr.	Species of Sialic Acids (% of Total Sialic Acids)		
	Neu5Ac	Mono- <i>O</i> -Ac -Neu5Ac	Oligo- <i>O</i> -Ac -Neu5Ac
uninvolved colonic mucosa			
13045	82.6	17.4	0
12599	57.7	37.9	4.4
11430	70.8	24.7	4.5
17672	83.3	16.7	0
14730	55.3	43.2	1.5
17421	82.6	8.9	8.5
16413	89.1	9.7	1.2
14445	66.3	20.7	13
25308	81.9	15.1	3.0
17960	84.7	10.9	4.4
25096	70.1	26.5	3.4
26547	62.9	31.8	6.2
26570	57.6	27.8	14.6
	72.6±	22.4±	5.0±
	11.9	10.8	4.6
cancerous colonic mucosa			
13045	92.6	7.4	0
12599	90.4	9.6	0
11430	76.7	21.9	1.8
17672	95.9	4.1	0
14730	88.1	11.9	0
17421	97.4	2.6	0
16413	-	-	-
14445	89.6	10.4	0
25308	97.4	2.6	0
17960	95.9	4.1	0
25096	92.2	7.8	0
26547	86.0	14	0
26570	60.7	39.3	0
	88.6±	11.3±	-
	10.5	10.4	

Moreover, as shown in Figure 3-38, the reduction in the level of *O*-acetylated sialic acids occurs not only in the cytosolic fraction (t-paired test, $p < 0.005$) but also in the membrane fraction (t-paired test, $p < 0.005$) from cancerous colonic mucosa. This is different from the

previous report that the cancerous colonic mucosa expressed a constant level of mono-*O*-acetylated sialic acids, which showed no significant difference from that observed in normal colonic mucosa [95]. The difference between these two investigations is supposed to be due to the cancerous tissue samples used representing different cancer stages.

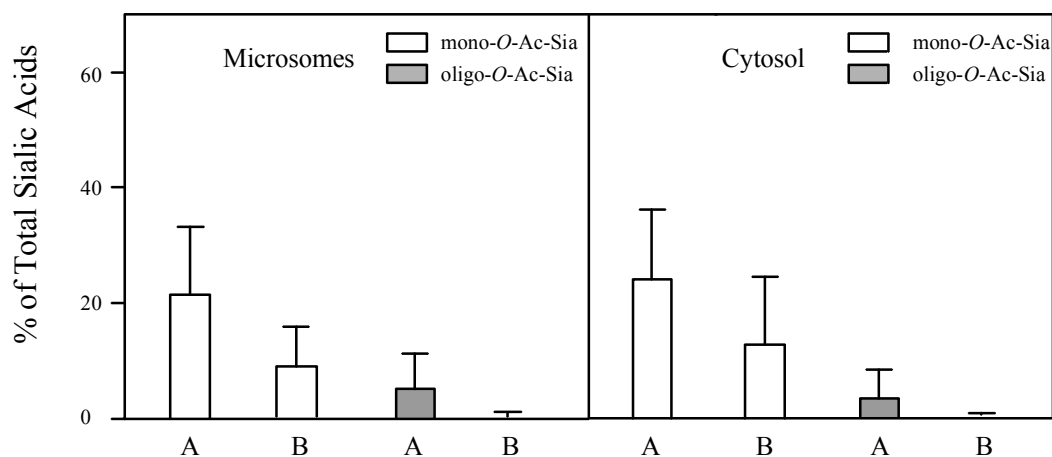


Figure 3-38 Relative Amount of *O*-Acetylated Sialic Acids in Subcellular Fractions from the Uninvolved and Cancerous Colonic Mucosa. Sialic acids in the cytosolic and microsomal fractions from uninvolved (A) and cancerous mucosa (B) were prepared, purified and analyzed using the procedure described in section 2.2.4, page 24. The results shown here were obtained from 12 matched tissue samples.

To perceive the relationship between the reduction extent of mono- and oligo-*O*-acetylated sialic acids in cancerous colonic mucosa and cancer stage, the ratio of the relative level of *O*-acetylated sialic acids in the cancerous mucosa to that in the uninvolved mucosa was plotted as a function of cancer stage. The cancer stage was judged by TNM classification system (appendix III) [104]: stage I to IV.

As shown in Figure 3-39, no apparent correlation was found between the cancer stage and the decreasing extent of mono- and oligo-*O*-acetylated sialic acids. Of 12 cancerous samples at stages II-IV, the significant reduction or complete loss of oligo-*O*-acetylated sialic acid was observed.

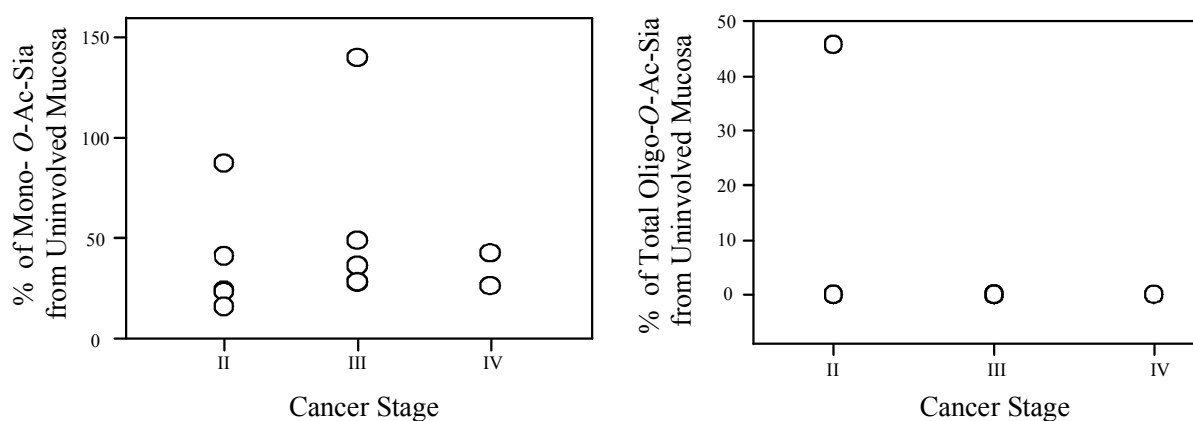


Figure 3-39 Relationship between the Decrease Extent of *O*-Acetylated Sialic Acids and Cancer Stage. The cancer malignancy was assessed by the TNM classification system. Of 12 samples investigated, 5 were in stage 2, 5 were in stage 3, and 2 were in stage 4.

3.6.2 The Reduction of OAT Activity in Cancerous Colonic Mucosa

To explore the underlying mechanism for the alteration of *O*-acetylated sialic acid level in cancerous mucosa, the activities of OAE and OAT in the uninvolved and cancerous mucosa from the same patient were determined and compared.

As shown in Figure 3-40, the activities of 9-OAE and oligo-OAE were observed in both the uninvolved mucosa and cancerous mucosa. Comparison of the activities of 9-OAE and oligo-OAE between cancerous and uninvolved colonic mucosa from the same patient shows no significant difference in enzyme activities in the cytosolic, microsomal and lysosomal fractions (t-paired test, $p > 0.05$).

In contrast to OAE, the activity of 7(9)-OAT against CMP-Neu5Ac in cancerous mucosa was significantly decreased (t-paired test, $p < 0.05$), when compared with that detected in the uninvolved mucosa from the same patient (Figure 3-41). Taken together, the results obtained here suggest that the decrease in the activity of 7(9)-OAT in cancerous colonic mucosa results in the reduction of *O*-acetylated sialic acid amount in cancerous tissues.

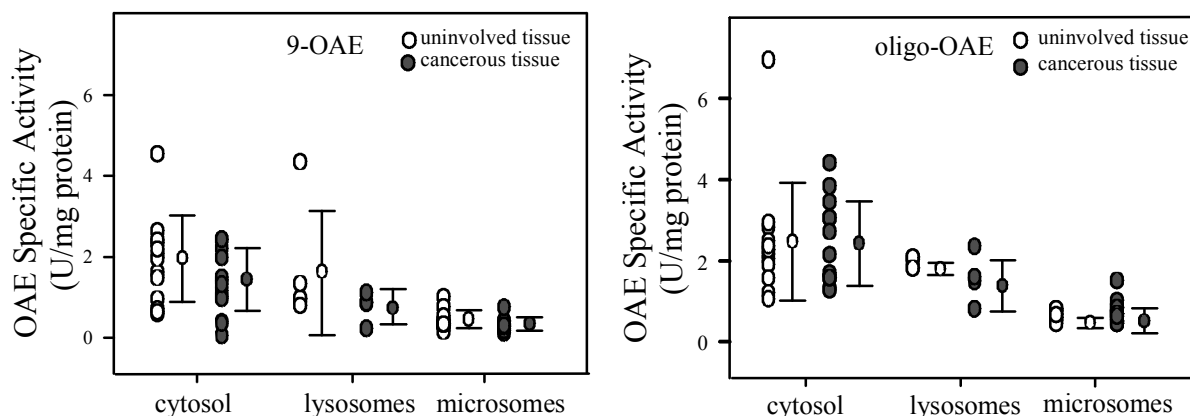


Figure 3-40 Comparison of the OAE Activities in Various Subcellular Fractions from the Uninvolved and Cancerous Colonic Mucosa. The activities of 9-OAE and oligo-OAE in the cytosol (n=12), microsomes (n=12) and lysosomes (n=5) were determined as described in section 2.2.14, page 35. The bars show the mean \pm SD.

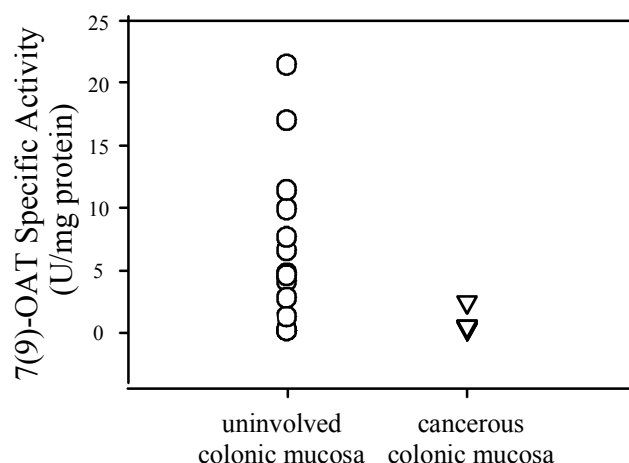


Figure 3-41 A Decrease in the Activity of 7(9)-OAT in Cancerous Colonic Mucosa. Using CMP-Neu5Ac as acceptor substrate, the activity of 7(9)-OAT in the uninvolved colonic mucosa (n=12) and cancerous mucosa (n=6) were measured as described in section 2.2.5, page 26.

3.6.3 Regulation of the Amount of Glycoconjugate-bound *O*-Acetylated Sialic Acids by OAT and Lse

Lse is proposed to participate in the degradation of *O*-acetyl groups present on sialoglycoconjugates in the lysosomal compartment [63], while OAT is responsible for the biosynthesis of *O*-acetylated sialic acids. Therefore, the relative activity of OAT to lysosomal

OAE might be responsible for the amount of total *O*-acetylated sialic acids associated with glycoconjugates.

Figure 3-42 shows that, in both the uninvolved and cancerous tissues, the higher ratio of the OAT/Lse activity corresponds to the higher level of glycoconjugate-bound *O*-acetylated sialic acids. A correlation coefficient of 0.8 (Spearman's rank correlation, appendix IV), for uninvolved or cancerous colonic mucosa, indicates a very good relationship between the two variables investigated. It thus suggests that the balance of OAT and Lse plays an important role in keeping the constant amount of glycoconjugate-bound *O*-acetylated acetylated sialic acids in human colonic mucosa.

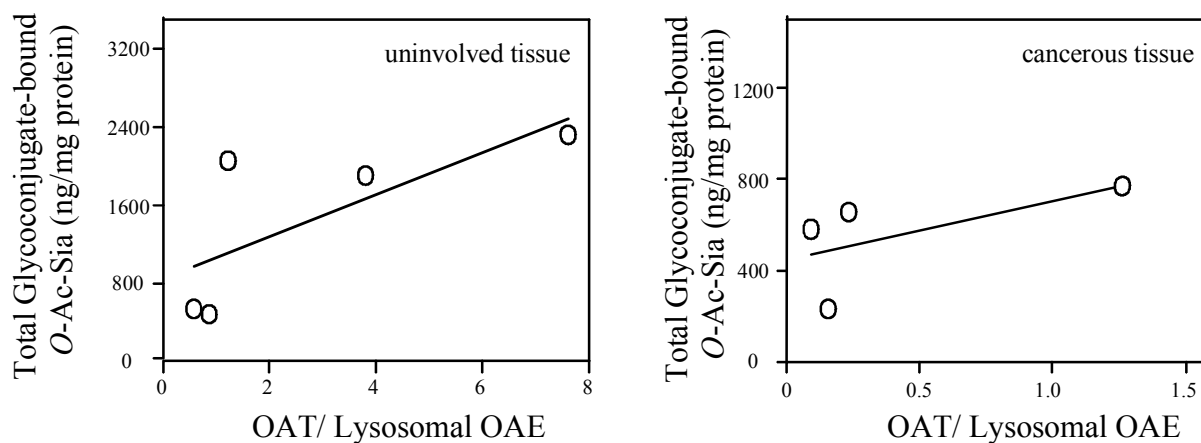


Figure 3-42 Correlation between the Amount of Glycoconjugate-bound *O*-Acetylated Sialic Acids and the Relative Activity of OAT to Lysosomal OAE. The amount of total glycoconjugate-bound *O*-acetylated sialic acids was plotted against the relative activity of OAT to lysosomal OAE. The line was fitted by regression analysis and the coefficient of correlation (r_s) for the two variables were determined by Spearman's rank correlation.

3.6.4 Regulation of the Free Cytosolic *O*-Acetylated Sialic Acid Amount by Cytosolic OAE

It has previously been proposed that cytosolic OAE is involved in the degradation of free *O*-acetylated sialic acids in the cytosol [63]. Therefore, the relationship between the amount of free *O*-acetylated sialic acids in the cytosol and the activity of cytosolic OAE was investigated. In the cytosolic fraction prepared from colonic mucosa, the level of oligo-*O*-acetylated sialic acids was quite low, therefore, only the amount of free mono-*O*-acetylated sialic acids in the cytosol was plotted against the activity of cytosolic 9-OAE.

As shown in Figure 3-43, the higher level of cytosolic 9-OAE activity was accompanied with the lower level of cytosolic free mono-*O*-acetylated sialic acids. For uninvolved colonic mucosa, the correlation coefficient was -0.35 (Spearman's rank correlation), suggesting that the function of cytosolic 9-OAE is to remove *O*-acetyl groups from free *O*-acetylated sialic acids. With regard to cancerous mucosa, the existence of free Neu5,9Ac₂ was not confirmed by GC-MS analyses, therefore, the relationship of the amount of cytosolic free mono-*O*-acetylated sialic acids and the level of cytosolic 9-OAE activity was not analyzed.

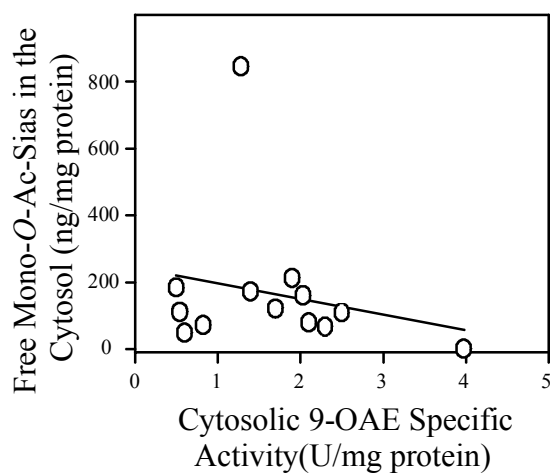


Figure 3-43 Relationship between Cytosolic 9-OAE Activity and the Amount of Cytosolic Free Mono-*O*-Acetylated Sialic Acids. The amount of cytosolic free mono-*O*-acetylated sialic acids was plotted against cytosolic 9-OAE activity. The line was fitted by regression analysis and the coefficient of correlation (r_s) for the two variables was determined by Spearman correlation rank.

4 Discussion

4.1 *O*-Acetylated Sialic Acids Level and Human Colorectal Cancer

4.1.1 The Nature of Sialic Acids in Human Colonic Mucosa

To estimate the patterns and relative amounts of *O*-acetylated sialic acids in human colonic mucosa, most analyses were limited to the periodate oxidation and colorimetric estimations of sialic acid concentration [121;139-142], paper chromatography [93;94], TLC [142] and HPLC [69;95]. Hutchins *et al.* (1988, 1988a) [93;94], however, confirmed the existence of mono-, di- and tri-*O*-acetylated sialic acids in normal human colonic mucosa and carcinoma cells by FAB-MS (fast atom bombardment-mass spectrometry) analysis. Those studies show that the level of *O*-acetylated sialic acids is up to 50 %, with above 30 % being oligo-*O*-acetylated sialic acids.

In the investigations reported here, the nature of sialic acids in the microsomal membrane and cytosolic fractions prepared from uninvolved human colonic mucosa (the excised end of colon tissue resected for carcinoma) was confirmed for the first time by GC-MS. *N*-Acetylneuraminic acid (Neu5Ac) and its *O*-acetylated derivatives were the only sialic acids observed. The presence of glycoconjugate-bound Neu5,9Ac₂ and Neu5,8,9Ac₃ in the cytosolic and membrane fractions, and of free Neu5,9Ac₂ in the cytosolic fraction were confirmed. The presence of Neu5,7,8,9Ac₄ in the membrane fraction was also confirmed. The similarity of the results obtained from fluorometric HPLC analysis and GC-MS shows the reliability of fluorometric HPLC analysis.

4.1.2 Reduction of Oligo-*O*-Acetylated Sialic Acids is an Early Event of Malignant Transformation in Human Colorectal Cancer

The expression of *O*-acetylated sialic acids in human colonic tissues shows racial variability [69], which is assumed to be the distribution of a single dominant gene regulating sialate *O*-acetylation (*oat*). About 9 % of the Caucasian population is homozygous (*oat*⁺*oat*⁺) for sialate *O*-acetylation, and this ratio is increased in Asians. In this case, the loss of *O*-acetylated sialic acids seems not to be related to any disease but is probably due to gene mutation resulting from the selection pressure [69]. With this exception, the reduction of *O*-acetylated sialic acids is believed to be a phenomenon occurring in the development of neoplasma [69;143].

It has been reported that the oligo-*O*-acetylated sialic acids in normal human colonic tissues is above 30 %, while the mono-*O*-acetylated sialic acids level ranges between 10-15 % [94;144]. When compared with these literature values, the uninvolved colonic mucosa investigated here expressed a relatively high proportion of mono-*O*-acetylated sialic acids (22.4 ± 10.8 %) but the lower level of oligo-*O*-acetylated sialic acids (5.0 ± 4.6 %). This phenomenon has been observed by other groups [94;95]. The abnormal pattern of *O*-acetylated sialic acids expressed in the uninvolved colonic mucosa, which are histologically normal, is supposed to be the result of a premalignant field defect, rather than a local secondary effect of tumor growth [145;146]. The reduced level of *O*-acetylation in the case of ulcerative colitis [147], a potentially premalignant state, supports this suggestion. Moreover, the significant reduction or loss of the oligo-*O*-acetylated sialic acids was observed in the 12 cancerous samples investigated, which represent stage II to IV. Taken together, our findings show that the reduction of oligo-*O*-acetylated sialic acids in human colonic mucosa is an early event during the malignant transformation in human colorectal cancer. The same phenomenon was previously observed in the cultured human colorectal cell lines at different stages of the adenoma-carcinoma sequence [95].

It was also found that cancerous mucosa expresses a significantly lower level of mono-*O*-acetylated sialic acids when compared with the uninvolved mucosa from the same patient (n=12). However, no apparent correlation was found between the cancer stage and the decreasing extent of mono-*O*-acetylated sialic acid.

4.2 Sialate *O*-Acetylation in Human Colonic Mucosa

4.2.1 Characterization of Membrane Sialate-7(9)-*O*-Acetyltransferase from Human Colonic Mucosa

Since the first discovery of the OAT in bovine submandibular glands more than 30 years ago [33;34], the OAT has been extensively investigated in rat liver [36;37;71;148], equine submandibular glands [6], guinea pig liver [5], and in K1-positive *Escherichia coli* [136] as well as Chinese Hamster Ovary cells [41]. However, little is known about the underlying enzymatic reaction which governs sialate *O*-acetylation in human colonic tissue, in spite of the fact that sialic acids in human colonic mucins are highly *O*-acetylated, and that a relationship between the loss of *O*-acetylation, as well as 7(9)-OAT activity, and colorectal cancer has been reported [95].

Utilizing microsomal membrane fractions isolated from human colonic mucosa, the 7(9)-OAT activity against a number of possible exogenous acceptor substrates and endogenous substrate was determined. Of all the substrates tested, CMP-Neu5Ac was found to be the best acceptor of acetyl groups transferred from AcCoA by OAT. Till now, CMP-Neu5Ac acting as an acceptor substrate for the OATs has never been reported. However, not only has 9-*O*-acetylated CMP-Neu5Ac previously been isolated from bovine submandibular glands [39], but a small, reproducible activity using CMP-Neu5Ac as the acceptor substrate has been observed in our laboratory against a soluble 7(9)-OAT activity from bovine submandibular glands (unpublished data).

The following observations provide evidence that CMP-Neu5Ac is acting as an acceptor substrate for a membrane-bound 7(9)-OAT from human colonic tissue. First, the incubation of microsomal protein with CMP-Neu5Ac and [³H]AcCoA resulted in the formation of Neu5,9Ac₂ and oligo-*O*-acetylated sialic acid. Both products can be saponified by alkaline treatment, with that corresponding to Neu5,9Ac₂ being susceptible to both the action of the 9-*O*-acetyl-specific esterase (sialate-9-*O*-acetylcyste-esterase) from the influenza C virus, as well as sialate-pyruvate-lyase.

The formation of oligo-*O*-acetylated sialic acids is not an entirely unexpected observation, since this sialic acid was detected by fluorometric HPLC analyses of the microsomal membrane fraction isolated from human colonic tissue. It is not clear whether the formation of oligo-*O*-acetylated sialic acids and Neu5,9Ac₂ is due to the action of two separate enzyme activities catalyzing the *O*-acetylation at C-7 and C-9 independently, or is due to the action of one OAT and a migrase that catalyzes the migration of acetyl groups from C-7 to C-8 or C-9, as was postulated to occur in bovine submandibular glands by Vandamme-Feldhaus and Schauer (1998) [35]. It must be noted that no Neu5,7Ac₂ was observed in this study following the incubation of microsomal protein with CMP-Neu5Ac and [³H]AcCoA, neither by radio-TLC nor radio-HPLC, and only a small amount of Neu5,7Ac₂ was detected by fluorometric HPLC analyses of various fractions isolated from human colonic mucosa (Table 3-1, page 45). This suggests that rapid migration of *O*-acetyl groups, followed by further *O*-acetylation, along the side chain (from C-7 to C-9) might result in the observation, using the techniques currently available, of only oligo-*O*-acetylated sialic acids and Neu5,9Ac₂. Only experiments utilizing a purified sialate-7(9)-OAT can further shed light on the complex enzymatic reaction that governs side chain sialate *O*-acetylation.

Further evidence for the enzymatic *O*-acetylation of CMP-Neu5Ac by human colonic 7(9)-OAT was afforded by the isolation of CMP-Neu5,9Ac₂, the putative product. This product, as

analyzed by radio-TLC on cellulose, was susceptible to alkaline treatment, as well as to mild acid hydrolysis, resulting in an increase in the peak intensity associated with Neu5,9Ac₂. The isolated putative CMP-Neu5,9Ac₂ was also capable of being utilized by the purified α 2,6-ST from rat liver, as well as by Golgi ST from human colonic mucosa. STs can only transfer sialic acid to an appropriate glycoconjugate acceptor from CMP-activated sialic acid [149]. However, modifications to the sialic acid moiety are tolerated, with a number of C-9-substituted CMP-Neu5Ac derivatives being found to act as suitable ST donor substrates [65;67;150]. Therefore, the finding that [³H]Neu5,9Ac₂ can be transferred from isolated CMP-[³H]Neu5,9Ac₂ to asialofetuin by rat liver and human colonic ST conclusively confirms that the product of the incubation of human colonic microsomes with CMP-Neu5Ac and [³H]AcCoA is CMP-Neu5,9Ac₂.

The characterization of the membrane-bound 7(9)-OAT from human colonic mucosa, using CMP-Neu5Ac as the exogenous acceptor, was investigated. Table 4-1 summarizes the properties of the OATs from human colonic tissues, rat liver [36], guinea pig liver [5], as well as bovine and equine submandibular glands [6;35;39]. It can be seen that with regard to temperature, temperature, pH, AcCoA affinity, as well as the subcellular localization, the colonic 7(9)-OAT shows similar properties to those described in other tissues. The activity of membrane OAT, including human colonic 7(9)-OAT is dependent on the membrane integrity, and can be competitively inhibited by CoA. Furthermore, the sialate *O*-acetylation in human colonic mucosa requires the involvement of histidine, lysine and free thiol residues. These residues have been also found to be essential for enzymatic sialate *O*-acetylation in rat liver [36] and bovine submandibular glands [35].

However, the 7(9)-OAT from human colonic tissue has one property that sets it apart, the ability to utilize, with a high affinity, CMP-Neu5Ac as an exogenous acceptor substrate. This finding, therefore, raises the question, what is the pathway by which sialate *O*-acetylation takes place in human colonic mucosa?

Table 4-1 Properties of the Sialate-*O*-Acetyltransferases from Various Sources

	Human Colonic Mucosa	Rat Liver [36]	Bovine Submandibular Gland [35]	Guinea Pig Liver [5]	Equine Submandibular Gland [6]
Attachment sites of <i>O</i> -acetyl groups	Side chain (C-7 or C-9)	Side chain (C-7 or C-9)	Side chain (C-7 or C-9)	Pyranose ring (C-4)	Pyranose ring (C-4)
Subcellular location	Golgi	Golgi	Golgi and cytosol ^a	Golgi	Fine-membrane fraction
Optimal pH	7	7	7	6.7	6.6
Optimal temp. (°C)	37	22	37	30	37
Acceptor substrate	CMP-Neu5Ac	endogenous	endogenous	endogenous	endogenous
K_M for AcCoA (μ M)	6.1	2.9	1.6	0.6	32.1
Inhibition by CoA	+ (K_i , 11.8 \pm 4.2 μ M)	+	+	+ (K_i 4.2 μ M)	+
Inhibition by <i>p</i> -CMB	+	+	+	Not detected	Not detected
Dependency on membrane integrity	+	+	+	+	+

^a7(9)-OAT activity in the cytosol was determined using free Neu5Ac as substrate [39]

4.2.2 Pathway of Sialate *O*-Acetylation in Human Colonic Mucosa Golgi Vesicles

Early studies using bovine and equine submandibular glands identified a cytosolic 7(9)-OAT activity that could transfer acetyl groups from AcCoA to either exogenous or endogenous free sialic acid [39;40]. In these studies it was proposed that the *O*-acetylation of sialic acids could occur at the level of free sialic acids, and that the enzyme product 9-*O*-acetylated Neu5Ac could be then activated to CMP-Neu5,9Ac₂, which can be utilized by STs in the microsomes from the same tissues [40;151]. The existence of a real cytosolic OAT is still an open question, however, these observations suggest that the sialate *O*-acetylation might also occur prior to its transfer to glycoconjugates.

On the contrary, more recent investigations on sialate *O*-acetylation in rat liver [36;37;152] suggest that, with the involvement of a trans-membrane transfer of acetate groups, the *O*-acetylation of sialic acids may take place within the lumen of the Golgi apparatus, after the transfer of sialic acids to glycoconjugates. Similar results were also obtained for the *O*-acetylation of gangliosides in the Golgi-enriched vesicles from human melanoma cells [38;72]. Even in bovine submandibular glands, the sialate *O*-acetylation occurring in the membrane fractions appears to take place after the transfer of sialic acids onto glycoproteins and glycolipids [35].

However, in human colonic mucosa, the pathway of sialate *O*-acetylation is different from those previously reported in several aspects [35-37;39;72]. First, the sialate *O*-acetylation occurs prior to the transfer of sialic acids onto glycoconjugates, however, the acetyl group is incorporated into CMP-Neu5Ac instead of free or glycosidically linked sialic acids. Second, human colonic 7(9)-OAT catalyzes the *O*-acetylation of CMP-Neu5Ac in the Golgi lumen, which requires the luminal pool of CMP-Neu5Ac and AcCoA.

CMP-Neu5Ac can be transported from the cytosol into the Golgi lumen by a CMP-Neu5Ac transporter, which can be inhibited by CMP and CMP-sialic acid derivatives [19;24;153]. It was found that preloading of Golgi vesicles with CMP-Neu5Ac for short times can result in an increase in the activity of colonic OAT, while selective inhibition of CMP-Neu5Ac translocation by CMP can lead to the reduction of enzyme activity. These observations indicate the dependency of enzyme activity on the Golgi luminal CMP-Neu5Ac concentration, which can be regulated by CMP-Neu5Ac transporter activity.

A putative transporter specific for AcCoA has been supposed to be essential for the *O*-acetylation of sialic acids present on glycoconjugates in rat liver [36] and bovine

submandibular glands [35]. In human colonic mucosa, the inhibition of AcCoA transport with CoA and DIDS, the two known inhibitors for AcCoA transporter [5;35;37], was found to be able to decrease 7(9)-OAT activity. This suggests that a similar AcCoA transporter is also required for sialate *O*-acetylation.

In nature, sialic acids are typically found at the terminal position of glycans on the glycoconjugates but not in free form. *In vivo*, the transfer of sialic acids to glycoproteins and glycolipids occurs in the Golgi compartment by sialyltransferases. In a functional Golgi compartment exist all the elements for sialylation, including sialyltransferases, the suitable glycoconjugate acceptors, as well as the specific transporter for the uptake and concentration of activated sialic acids [128;153;154]. *In vitro*, intact and right-orientated Golgi vesicles prepared from fresh chilled tissues are believed to contain all these elements, and thus can be used for mimicking *in vivo* sialylation by labeling the endogenous glycoprotein acceptors in the Golgi vesicles with radiolabeled CMP-linked sialic acids [152;155]. It was observed that, upon the incubation of Golgi vesicles with [³H]AcCoA and CMP-Neu5Ac, [³H]Neu5,9Ac₂ can be directly transferred from the neo-synthesized CMP-[³H]Neu5,9Ac₂ to endogenous glycoconjugates in human colonic mucosa Golgi vesicles. This suggests that sialyltransferase(s) is involved into the expression of *O*-acetylated sialic acids on glycoconjugates in colonic mucosa.

The formation of CMP-linked oligo-*O*-acetylated sialic acids, though not confirmed, is most likely since a certain amount of oligo-*O*-acetylated sialic acids is formed following the incubation of membrane proteins with AcCoA and CMP-Neu5Ac. It is also observed that the incubation of AcCoA with microsomal proteins resulted in the incorporation of acetyl group into the glycoconjugates. However, it is difficult to distinguish whether the acetyl group is directly incorporated into glycoconjugate-bound sialic acids by another OAT in the human colonic Golgi vesicles, or the acetyl groups is first incorporated into endogenous CMP-Neu5Ac, and the newly produced CMP-Neu5,9Ac₂ is utilized by the STs in the same tissue to sialylate to the endogenous glycoconjugates. These questions can only be clarified when the purification of the enzyme is achieved.

As summarized in Figure 4-1, in colonic mucosa, the expression of *O*-acetylated sialic acids present on glycoconjugates involves multiple-step reactions. First, the cytosolic CMP-Neu5Ac and AcCoA are transported into the Golgi lumen by CMP-Neu5Ac and putative AcCoA transporters, respectively. In the Golgi lumen, sialate-7(9)-OAT catalyzes the incorporation of the acetyl group from AcCoA into CMP-Neu5Ac, resulting CMP-

Neu5,9Ac₂. Finally, at the action of human colonic STs, Neu5,9Ac₂ can be transferred from the neo-synthesized CMP-Neu5,9Ac₂ to certain glycoconjugate acceptors.

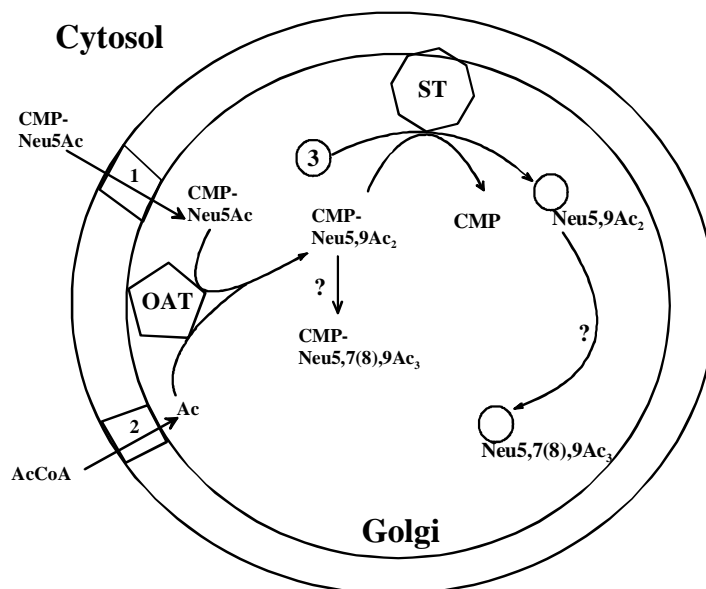


Figure 4-1 Scheme of the Pathway for Sialate *O*-Acetylation in Human Colonic Mucosa. 1-CMP-Neu5Ac transporter; 2-AcCoA transporter; 3-glycoprotein.

4.2.3 A Sialyltransferase Preferring to Utilize CMP-Neu5,9Ac₂ as the Donor Substrate to Sialylate Certain Acceptors ?

Previous investigations on purified STs showed that these enzymes can utilize a number of CMP-Neu5Ac substitutes as donor substrate, however, with low efficiency [65;67;150]. The transfer of 9-*O*-acetylated Neu5Ac relative to Neu5Ac has been shown to range from 75 % for the bovine Galβ1,4GlcNAc-α2,6-sialyltransferase to only 10 % for the bovine GalNAc-α2,6-sialyltransferase, even though the acceptor substrate used are corresponding to the specificity of the ST investigated [72;150]. These observations suggest that those STs have donor substrate specificity for CMP-Neu5Ac.

In this investigation, it is found that human colonic STs can not only directly transfer Neu5,9Ac₂ from neo-synthesized CMP-[³H]Neu5,9Ac₂ to endogenous glycoconjugates in the same Golgi vesicles, but also showed a preference for utilizing CMP-Neu5,9Ac₂ over CMP-Neu5Ac to sialylate asialofetuin. These results suggest that there exists at least one ST having the donor substrate preference for CMP-Neu5,9Ac₂ in human colonic mucosa.

However, the incorporation rate of Neu5Ac into asialo-BSM by human colonic STs was much faster than that observed for Neu5,9Ac₂. The difference between asialofetuin and asialo-BSM as the acceptor for Neu5,9Ac₂ indicates that the incorporation of Neu5,9Ac₂ into glycoconjugates is dependent on not only the synthesis of CMP-Neu5,9Ac₂ but also the existence of a suitable acceptor. A comparison of the oligosaccharide structures present on fetuin and BSM (Table 4-2) suggests that GalNAc α - is not the optimal acceptor for Neu5,9Ac₂, while Gal β 1,4GlcNAc β - or/and Gal β 1,3GalNAc α - appear to be the better acceptors.

Table 4-2 Main Neutral Oligosaccharide Structures in Human Colonic Mucin, Bovine Submandibular Gland Mucin and Fetuin.

Protein	Oligosaccharide Structure
HCM [82;83].	GalNAc α -Ser/Thr
	GlcNAc β 1,3GalNAc α -Ser/Thr
	Gal β 1,4GlcNAc β 1,3GalNAc α -Ser/Thr
	GlcNAc β 1,3Gal β 1,4 GlcNAc β 1,3GalNAc α -Ser/Thr
	Gal β 1,4GlcNAc β 1,3Gal β 1,4GlcNAc β 1,3GalNAc α -Ser/Thr
BSM [119;156;157]	GalNAc α -Ser/Thr (53.3 %)
	GlcNAc β 1,3GalNAc α -Ser/Thr (22.3 %)
Fetuin [67]	Gal β 1,4GlcNAc β -
	Gal β 1,3GalNAc α -Ser/Thr

In human colonic mucosa, the location of *O*-acetylated sialic acid in glycoconjugates has not been established. However, it has been demonstrated that HCMs possess high levels of *O*-acetylated sialic acids, and the terminal sequence Gal β 1,4GlcNAc β -. Therefore, it seems that HCMs are the specific acceptor for the human colonic ST specific for CMP-Neu5,9Ac₂.

Nevertheless, the final conclusion can only be made when the assumed ST(s) is purified, and the location of *O*-acetylated sialic acid in the glycoconjugates synthesized in human colonic tissue is established.

Previous investigations on OATs suggest that there seems to be more than one distinct sialate-*O*-acetyltransferases in mammals, with one *O*-acetylating α 2,6-linked sialic acids of *N*-linked sialoglycoproteins [41;71], and another *O*-acetylating α 2,8-linked sialic acids of gangliosides [41]. Our findings suggest that there might exist a third enzyme for the *O*-acetylation of sialic acids on oligosaccharides of mucin-type proteins through the formation of a CMP-Neu5,9Ac₂ intermediate, which can be further transferred to a certain glycoconjugate, presumably human colonic mucins, by a ST preferring to use CMP-Neu5,9Ac₂ as the donor substrate.

4.3 Sialate De-*O*-Acetylation in Human Colonic Mucosa

Human colonic tissue is characterized by its high level of *O*-acetylated sialic acids [82;83;92-96]. Since *O*-acetyl esters can hinder or abolish the action of sialidase and sialate-pyruvate-lyase [55;66;75], it is reasonable to predict the existence of OAE in human colonic tissue. Actually, OAE activity has been detected in human faecal extracts [52] and in many individual human enteric bacterial strains [158], however, very little is known about the turnover and catabolism of the *O*-acetyl groups in human colonic mucosa.

Using free *O*-acetylated sialic acids as substrates, we have demonstrated the activity of OAE in colonic mucosa. The OAE activities are found to be localized either in the cytosolic fraction or the membrane fraction, probably, the lysosomal compartment. These results do not confirm, but imply that two forms of OAEs with distinct structural properties are present in human colonic mucosa, similar to that observed in rat liver and rat colonic mucosa [53;56]. One is a cytosolic non-glycosylated OAE (Cse), and the other is a membrane-associated glycosylated OAE (Lse) [53;56].

In mammals, most OAEs appear to be highly specific for the cleavage of 9-*O*-acetyl groups from sialic acids [53;55]. With the aid of fluorometric HPLC analysis, human colonic OAEs are found to be specific for the hydrolysis of 9-*O*-acetyl groups from sialic acids, and the complete de-*O*-acetylation of sialic acids can be achieved in a sequential manner. As shown in Figure 4-2, the hydrolysis of di-*O*-acetylated sialic acids first occurs at the 9-position of sialic acids, which results in the formation of Neu5,7Ac₂ and Neu5,8Ac₂. The migration of *O*-acetyl

group from 7- or 8- to 9-position allows the further degradation of these acetyl groups. This observation is in agreement with the previous investigation on rat liver [53].

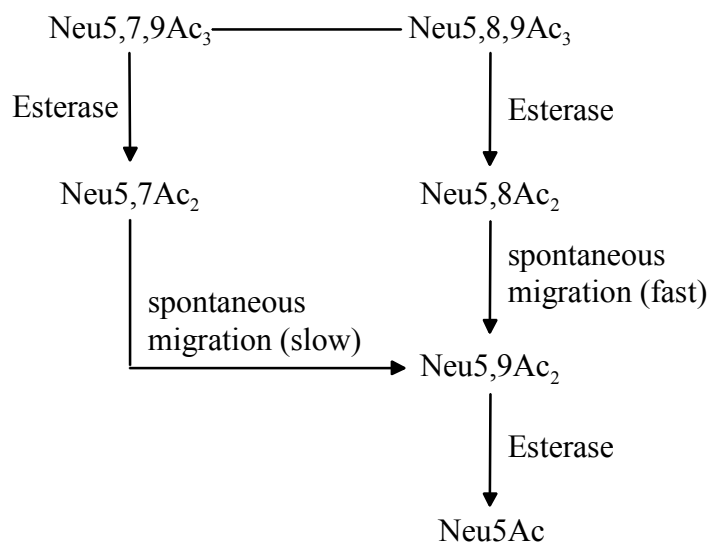


Figure 4-2 Proposed Scheme for Sequential Hydrolysis of Di-*O*-Acetylated Sialic Acids by Human Colonic OAE.

4.4 Regulation of the Expression Level of *O*-Acetylated Sialic Acids in Human Colonic Mucosa

The findings in this study suggest that different factors are involved in the regulation of the expression level of *O*-acetylated sialic acids on glycoconjugates in human colonic mucosa. These factors might include the regulation of OATs expression, the control of CMP-Neu5Ac and AcCoA transport, the regulation of a CMP-Neu5,9Ac₂-specific ST expression, the regulation of HCMs synthesis, and the control of *O*-acetylated sialic acids degradation. Among those factors, the balance between OAE and OAT is supposed to be the first potential regulation point of the expression of *O*-acetylated sialic acids.

The investigation of the relationship between the level of *O*-acetylated sialic acids and the activities of 7(9)-OAT and OAE in colonic mucosa (12 individual tissue samples) shed light on the roles of these enzymes in the regulation of *O*-acetylation. In human colonic mucosa, the higher observed Cse activity is associated with the lower amount of cytosolic free *O*-acetylated sialic acids. This observation indicates that Cse is involved in the hydrolysis of *O*-acetyl groups from cytosolic free *O*-acetylated sialic acids. These molecules are poor substrates for the CMP sialic acid synthase [150] and the existence of *O*-acetyl esters hinders

the action of sialate-pyruvate-lyase [66;75]. Therefore, it is proposed that the physiological function of cytosolic OAE is to facilitate further metabolism of free *O*-acetylated sialic acid in the cytosol.

In contrast to Cse, the Lse is believed to act on *O*-acetyl groups of sialoglycoconjugates to facilitate the action of sialidases, which is localized in the lysosomal compartment [2;8]. We found that the amount of glycosidically linked *O*-acetylated sialic acids in human colonic mucosa is coordinated well with the relative activity of OAT to lysosomal OAE. This indicates that the balance of these two enzymes plays a key role in keeping the normal level of glycosidically linked sialic acids in human colonic mucosa.

Moreover, the comparison of the activities of OAT and OAE in the uninvolved and cancerous mucosa from the same patient showed that a significant reduction in 7(9)-OAT activity occurs in the cancerous tissues. On the contrary, no significant difference was detected between the OAE activities, neither cytosolic OAE nor membrane OAE, in the uninvolved and cancerous mucosa. Therefore, it is proposed that a reduction in 7(9)-OAT activity, or more exactly, a changing in the balance of OAT and OAE, is one of the main reasons for the decrease in the level of sialate *O*-acetylation in cancer tissues.

5 Summary

The two main enzymes involved in the metabolism and catabolism of *O*-acetylated sialic acids are acetyl-coenzyme A:sialate-*O*-acetyltransferase (OAT) for the *O*-acetylation of sialic acids, and sialate-*O*-acylesterase (OAE) for the de-*O*-acetylation of *O*-acetylated sialic acids. The sialic acids present in human colonic mucosa are highly *O*-acetylated, and the decreasing expression level of *O*-acetylation has been demonstrated to be the result of malignant transformation in human colorectal cancer. However, little is known about the enzymatic processes for sialate *O*-acetylation and de-*O*-acetylation in this tissue, and the underlying mechanism for the alteration of *O*-acetylated sialic acid level in cancerous colonic mucosa is not yet clarified.

A membrane-bound sialate-7(9)-*O*-acetyltransferase (7(9)-OAT) has been found in human colonic mucosa. This enzyme is different from other reported OATs [5;6;35;37;136], for it utilizes CMP-Neu5Ac instead of glycoconjugate-bound sialic acid as the acceptor substrate. With the aid of chromatographic and enzymatic techniques, the main *O*-acetylated sialic acid formed is verified to be 9-*O*-acetylated Neu5Ac, and a significant amount of oligo-*O*-acetylated Neu5Ac is also synthesized. Moreover, the utilization of CMP-Neu5Ac as the acceptor substrate was confirmed by the isolation and characterization of the putative product, CMP-Neu5,9Ac₂, using ion-exchange chromatography. The ability of CMP-Neu5,9Ac₂ to act as a sialic acid donor for sialyltransferases affords the conclusive demonstration for the formation of CMP-Neu5,9Ac₂. Using CMP-Neu5Ac, the most efficient acceptor substrate of all those tested, the 7(9)-OAT activity was found to be optimal at 37 °C, with a pH optimum of 7.0. The activity of 7(9)-OAT shows the dependence on protein, CMP-Neu5Ac (K_M : 59.2 μM) and AcCoA (K_M : 6.1 μM) concentrations, as well as membrane integrity. The enzyme activity could be inhibited by CoA with a K_i of 11.9 μM. Subcellular fractionation and protease digestion shows that 7(9)-OAT is localized in the Golgi lumen.

In human colonic mucosa, the biosynthesis of CMP-Neu5,9Ac₂ appears to occur in the Golgi lumen. CMP-Neu5Ac and AcCoA are required to be transported from cytosol into the Golgi lumen to act as substrates for 7(9)-OAT. The Neu5,9Ac₂ can be directly transferred from the neo-synthesized CMP-Neu5,9Ac₂ to the endogenous glycoconjugates in the same Golgi vesicles. This suggests the existence of a ST preferring to utilize CMP-Neu5,9Ac₂ as the donor substrate.

Moreover, asialofetuin is found to be a much more efficient acceptor for Neu5,9Ac₂ than asialo-BSM, which shows that Neu5,9Ac₂ can be only transferred to certain glycoconjugates.

The comparison of the oligosaccharide sequences on fetuin and human colonic mucins shows that both contain the terminal sequence of Gal β 1,4GlcNAc β -. This suggests that human colonic mucins seem to be the appropriate acceptor substrate for the ST having donor substrate preference of CMP-Neu5,9Ac₂

The expression of *O*-acetylated sialic acids is remarkably tissue- and molecule-specific, and is developmentally regulated in various systems [4;7;8]. Investigations on OATs, together with the analyses of naturally occurring sialic acids, suggest that there must be more than one distinct sialate-*O*-acetyltransferase in mammals, with one *O*-acetylating α 2,6-linked sialic acids of *N*-linked sialoglycoproteins [41;71], and another *O*-acetylating α 2,8-linked sialic acids of gangliosides [41]. The data shown here suggest that there might exist a third enzyme for the *O*-acetylation of sialic acids on oligosaccharides of mucin-type proteins through the formation of a CMP-Neu5,9Ac₂ intermediate, which can be further transferred to certain glycoconjugates, presumably mucin-type proteins, by a ST preferring to use CMP-Neu5,9Ac₂ as the donor substrate. It is a pathway different from that observed in rat liver and human melanoma cells, which takes place after the transfer of sialic acids onto glycoconjugates in the Golgi lumen [37;38].

In this study, the activity of sialate-*O*-acetyl esterase (OAE) has been demonstrated in the cytosolic and lysosomal fractions prepared from human colonic mucosa. The OAEs are specific for the cleavage of the 9-*O*-acetyl groups from Neu5,9Ac₂ or Neu5,7(8),9Ac₃. In the latter case, complete de-*O*-acetylation takes place by sequential enzyme cleavage and intermediate migration of ester groups from 7- or 8- to 9-position. Moreover, the data presented here suggest that cytosolic OAE seems to be involved in the de-*O*-acetylation of free *O*-acetylated sialic acids in the cytosol, while lysosomal OAE might play an important role in the metabolism of *O*-acetyl groups on glycosidically bound sialic acids.

A comparison of the expression level of *O*-acetylated sialic acids and the activities of OAT and OAE in the uninvolved and cancerous mucosa from the same patient (n=12) shows a decrease in the level of *O*-acetylated sialic acid (both mono-*O*-acetylated and oligo-*O*-acetylated) and in the activity of OAE. However, no difference was observed between the activities of cytosolic and lysosomal OAE in the uninvolved and cancerous mucosa (p>0.05). These results suggest that the change of OAT activity but not the OAE activity results in the decrease in the expression level of *O*-acetylated sialic acids in cancerous colonic mucosa.

6 Zusammenfassung

Die in den Metabolismus und Katabolismus *O*-acetylierter Sialinsäuren involvierten beiden Hauptenzyme sind die Acetyl-Coenzym A:Sialat-*O*-acetyltransferase (OAT) für die *O*-Acetylierung von Sialinsäuren und die Sialat-*O*-acetyltransferase (OAE) für die De-*O*-acetylierung von *O*-acetylierten Sialinsäuren. Die in der humanen Darmmucosa vorkommenden Sialinsäuren sind stark *O*-acetyliert, und ein verringertes Niveau der *O*-Acetylierung wurde als Ergebnis einer malignen Transformation bei der Entstehung von colorectalen Tumoren beim Menschen beschrieben. Es ist jedoch sehr wenig über die enzymatischen Prozesse der Sialinsäure *O*-Acetylierung und De-*O*-acetylierung in diesem Gewebe bekannt und der zugrunde liegende Mechanismus der Veränderung des Niveaus der *O*-Acetylierung in entarteter humaner Darmmucosa ist bislang nicht aufgeklärt.

Eine membran-gebundene Sialat-7(9)-*O*-acetyltransferase (7(9)-OAT) wurde in humaner Darmmucosa gefunden. Dieses Enzym ist insofern von den bisher beschriebenen OATs [5;6;35;37;136] verschieden, als es CMP-Neu5Ac anstelle von glycoconjugate-gebundener Sialinsäure als Substrat benutzt. Die Eigenschaften der gebildeten *O*-acetylierten Sialinsäure wurde mit Hilfe von chromatographischen und enzymatischen Techniken bestätigt. So ist Neu5,9Ac₂ die häufigste *O*-acetylierte Sialinsäure, aber auch oligo-*O*-acetylierte Sialinsäuren wurden in signifikanten Mengen gefunden. Darüber hinaus wurde CMP-Neu5Ac als Acceptorsubstrat durch die Isolierung und Charakterisierung des vermutlichen Produktes, von CMP-Neu5,9Ac₂, durch Ionen-Austausch-Chromatographie bestätigt. Die Möglichkeit, das Sialyltransferasen CMP-Neu5,9Ac₂ als Sialinsäure-Donor nutzen können, erfordert den eindeutigen Nachweis der Bildung dieses Substrates. Mit CMP-Neu5Ac, dem von allen getesteten effizientesten Acceptorsubstrat, war die Aktivität der 7(9)-OAT optimal bei 37 °C und hatte ein pH-Optimum von 7,0. Die Aktivität der 7(9)-OAT war sowohl abhängig von der Proteinmenge, der CMP-Neu5Ac- (K_M : 59,2 μ M) und der AcCoA-Konzentration (K_M : 6,1 μ M) als auch von der Integrität der Membranen. Die Enzymaktivität konnte durch CoA mit einem K_i von 11,9 μ M inhibiert werden. Subzelluläre Fraktionierungen und Proteaseverdaue zeigten die Lokalisierung der 7(9)-OAT im Golgi-Lumen.

In humaner Darmmucosa scheint die Biosynthese von CMP-Neu5,9Ac₂ im Golgi-Lumen abzulaufen. CMP-Neu5Ac und AcCoA müssen daher aus dem Cytosol in das Golgi-Lumen transportiert werden, um dort als Substrate der 7(9)-OAT zur Verfügung zu stehen. Neu5,9Ac₂ kann dort von dem neu synthetisierten CMP-Neu5,9Ac₂ durch Sialyltransferasen auf endogene Glycoconjugate übertragen werden. Asialofetuin ist das deutlich effizientere Acceptorsubstrat für Neu5,9Ac₂ im Vergleich zu asialo-BSM, was darauf hindeutet, dass

Neu5,9Ac₂ nur auf bestimmte Glycoconjugate übertragen werden kann. Ein Vergleich der Oligosaccharid-Sequenzen von Fetuin und humanem Darmmucin zeigt, dass in beiden die terminale Sequenz Galβ(1,4)GlcNAcβ- vorliegt. Dies lässt vermuten, dass humanes Darmmucin das entsprechende natürliche Acceptorsubstrat der Sialyltransferasen ist, deren Sialinsäure-Donor CMP-Neu5Ac in diesem Gewebe ist.

Die Expression *O*-acetylierter Sialinsäuren ist bemerkenswert Gewebe- und Molekülspezifisch und ist in verschiedenen Systemen entwicklungs-abhängig reguliert [4;7;8]. Zusammen mit den Analysen natürlich vorkommender Sialinsäuren zeigen Untersuchungen von OATs, dass es verschiedene Sialat-*O*-acetyltransferasen in Säugetieren geben muss, eine für *O*-acetylierte Sialinsäuren in α(2,6)-Verknüpfung auf *N*-glycosylierten Sialoglycoproteinen [41;71] und eine andere für *O*-acetylierte Sialinsäuren in α(2,8)-Verknüpfung auf Gangliosiden [41]. Die hier gezeigten Daten deuten darauf hin, dass möglicherweise ein drittes Enzym für die *O*-Acetylierung von Sialinsäuren auf Oligosacchariden von Proteinen des Muzintyps existiert, mit einer zwischenzeitlichen Bildung von CMP-Neu5,9Ac₂, welches dann auf die Glycoconjugate des Muzintyps durch Sialyltransferasen, die dieses Donorsubstrat bevorzugen, übertragen wird. Dies ist ein anderer Weg als der in Rattenleber und humanen Melanomzellen beobachtete, wo die *O*-Acetylierung erst nach einer Übertragung der Sialinsäuren auf die Glycoconjugate im Golgi-Lumen stattfindet [37;38].

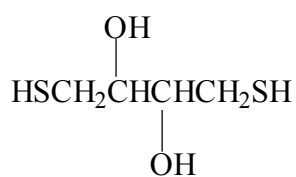
In dieser Untersuchung konnte die Aktivität einer Sialat-*O*-acetylsterase (OAE) in den cytosolischen und lysosomalen Fraktionen von humaner Darmmucosa nachgewiesen werden. Die OAEs spalten spezifisch die 9-*O*-Acetyl-Reste von Neu5,9Ac₂ oder Neu5,7(8),9Ac₃ ab. Im letzteren Fall kommt es durch einen sequentiellen enzymatischen Abbau der *O*-Acetyl-Reste von Position 9 mit anschließender Migration der verbleibenden Reste von den Positionen 7 bzw. 8 nach Position 9 zu einer vollständigen De-*O*-acetylierung. Darüber hinaus deuten die Daten darauf hin, dass cytosolische OAE für die De-*O*-acetylierung von freien Sialinsäuren im Cytosol verantwortlich sind, während die lysosomalen OAE eine wichtige Rolle im Metabolismus der *O*-Acetyl-Reste von glycosidisch gebundenen Sialinsäuren spielen.

Ein Vergleich der Expression von *O*-acetylierten Sialinsäuren und der Aktivitäten von OAT und OAE in nicht-kanzerösem und kanzerösem Darmgewebe derselben Patienten (n=12) zeigt einen Rückgang der *O*-acetylierten Sialinsäuren (sowohl mono- als auch oligo-*O*-acetyliert) und auch der Enzymaktivität der OAE. Es konnte jedoch kein Unterschied zwischen den Enzymaktivitäten der cytosolischen und der lysosomalen OAE in nicht-kanzerösem und kanzerösem Darmgewebe nachgewiesen werden (p>0,05). Diese Ergebnisse zeigen, dass

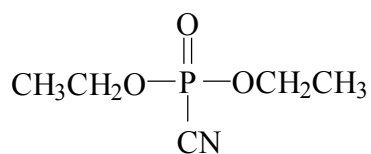
eine Veränderung der OAT-Aktivität, aber nicht der OAE-Aktivitäten, für den Rückgang der Expression von *O*-acetylierten Sialinsäuren in kanzeröser Darmmucosa verantwortlich ist.

Appendixes

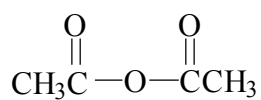
Appendix I Structures of Amino Acid-Modifying Reagents Used in the Inhibition Experiments



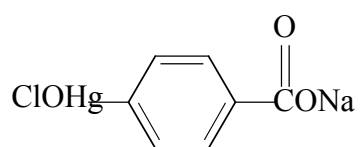
DTT



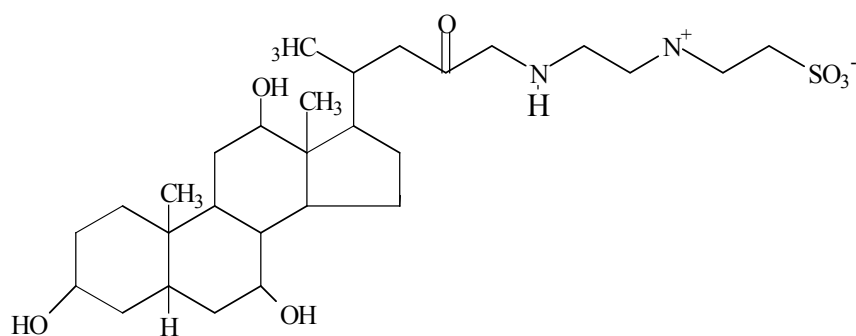
DEPC



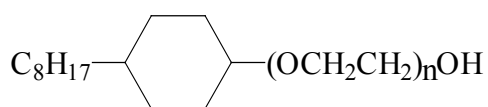
acetic acid anhydride

*p*-CMB

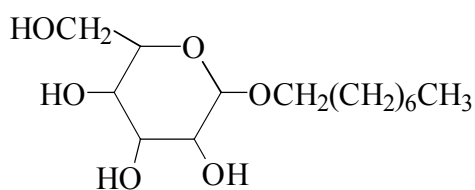
Appendix II Structures of Detergents Used in Solubilization



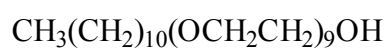
CHAPS



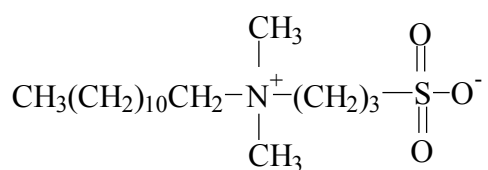
Triton X-100



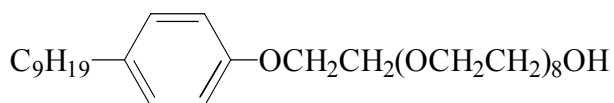
Octylglucoside



LUBROL Px



Zwittergent 3-12



Nonidet P40

Appendix III TNM Classification of Colorectal Cancer Stage

The American Joint Committee on Cancer (AJCC) has designated staging by TNM classification. TNM definitions:

- **Primary tumor (T)**

TX: Primary tumor cannot be assessed.

T0: No evidence of primary tumor.

Tis: Carcinoma *in situ*: intraepithelial or invasion of the lamina propria.

T1: Tumor invades submucosa.

T2: Tumor invades muscularis propria.

T3: Tumor invades through the muscularis propria into the subserosa, or into non-peritonealized pericolic or perirectal tissues.

T4: Tumor directly invades other organs or structures, and/or perforates visceral peritoneum.

- **Regional lymph nodes (N)**

NX: Regional nodes cannot be assessed.

N0: No regional lymph node metastasis.

N1: Metastasis in 1 to 3 regional lymph nodes.

N2: Metastasis in 4 or regional lymph nodes.

- **Distant metastasis (M)**

MX: Distant metastasis cannot be assessed.

M0: No distant metastasis.

M1: Distant metastasis.

Stage	TNM
0	Tis, N0, M0
I	T1, N0, M0 /T2, N0, M0
II	T3, N0, M0/ T4, N0, M0
III	Any T, N1, M0/ Any T, N2, M0
IV	Any T, Any N, M1

Appendix IV Spearman's Rank Correlation^[a,b]

Spearman's rank correlation is a correlation analysis suitable for non-Gaussian population. It works by assigning a rank to each observation in each group separately. Then calculate the sums of the squares of the differences in paired ranks(d_i^2) according to the formula:

$$R_s = 1 - 6 * (d_1^2 + d_2^2 + \dots + d_n^2) / [n(n^2 - 1)]$$

in which n is the number of observations.

R_s is the correlation coefficient, which ranges from -1 to 1 and shows the correlation of the variables.

R_s	Interpretation
$R_s = 0$	The two variables do not vary together at all.
$0 < R_s < 1$	The two variables tend to increase or decrease together.
$R_s = 1$	Perfect positive correlation.
$-1 < R_s < 0$	One variable increases as the other decreases.
$R_s = -1$	Perfect negative or inverse correlation.

a- <http://www.graphpad.com>; b-<http://nimitz.mcs.kent.edu>

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