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The roles of enteric bacterial sialidase, sialate *O*-acetyl esterase and glycosulfatase in the degradation of human colonic mucin

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Sialidase activity in normal faecal extracts showed a preference for mucin-related glycoprotein and oligosaccharide substrates, but the presence of two or more *O*-acetyl esters at positions C_7–C_9 on the sialic acids retarded the rate of hydrolysis. A specific sialate *O*-acetyl esterase was detected with a lower total activity relative to sialidase with mucin substrates and having a pH optimum of 7.8 and a K_M of approximately 1 mM sialate *O*-acetyl ester. A specific glycosulfatase activity was found in faecal extracts using the substrate lactit-[^{3}H]ol 6-*O*-sulfate with a pH optimum of pH 5.0 and a K_M of approximately 1 mM.

Faecal extracts from ulcerative colitis (UC) patients had higher sialate *O*-acetyl esterase and glycosulfatase activity, while mucin sialidase activity was unchanged.

Metabolically labelled mucin isolated from UC patients contained less sulfate and had lower sialic acid *O*-acetylation compared with normal mucin. Colonic mucin was degraded more efficiently by faecal extracts from UC patients compared with normal extracts. The UC mucin was degraded more rapidly than the normal mucin by faecal enzyme extracts from both normal and UC subjects.

Keywords: sialic acid, sialidase, sialate *O*-acetyl esterase, glycosulfatase, colonic mucin, enteric bacteria, ulcerative colitis

Abbreviations: UC, ulcerative colitis; BSM, bovine submandibular gland mucin; PMSF, phenylmethylsulfonyl-fluoride. Sialic acids are abbreviated according to Schauer [37].

The supramucosal layer covering the surface of the human colonic mucosa is a dynamic system being maintained by continual mucosal cell synthesis and degraded by a variety of aggressive factors, including the enzymes secreted by the normal enteric bacterial flora [1–3]. The layer is responsible for important defensive functions, and is made up of many components, of which the mucus glycoproteins (mucins) are predominant and give the secretion its characteristic viscoelastic properties [1, 2].

The mucus glycoproteins secreted by the human colonic mucosa are typically rich in sialic acids and sulfate [1, 3]. The sialic acids are *O*-acetylated at positions C_7–C_9 [4–6], while the location of the *O*-sulfate esters in the oligosaccharide chain is unknown [7–9]. The presence of these peripheral modifications in mucin oligosaccharides has been linked with the rate of mucin degradation by bacterial enzymes [1, 3, 8].

Sialidase activity is widely spread throughout the normal colonic bacterial flora [10] and is readily detected in faecal extracts [11]. Many bacterial sialidases have been detected and their general properties reported [12], but this group of bacterial enzymes has not been studied in detail and no individual sialidase has yet been purified. Roles for these enzymes in health and disease have been identified, and a physiologically significant nutritional function proposed for the normal human colon [13]. An inhibitory effect of substrates containing sialic acids *O*-acetylated at C_7–C_9 is known for purified [14] and enteric bacterial supernate [10] enzymes. However, the response of the total faecal sialidase has not been measured to assess the physiological significance of this sialic acid *O*-acetylation in colonic mucins. Enzymatic removal of the *O*-acetyl esters may take place as a result of sialate *O*-acetyl esterase activity which has been detected in human faecal extracts [11] and in many individual human enteric

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bacterial strains [10]. The total amount of esterase activity available for action on colonic mucus is also unknown.

Glycosulfatase activity has also been described in faecal extracts and for individual bacterial strains [10, 15–17]. The lack of structural data on human colonic mucin oligosaccharide sulfation has restricted an understanding of the relative efficiency of sulfatase activity with defined substrates and of any sialidase (glycosidase) and sulfatase interactions which may exist [15–17].

The defensive and exchange functions at the mucosal surface rely on an intact supramucosal barrier [1, 2], while degradation of the mucins in the layer provides a significant source of nutrition for the whole bacterial flora [18]. The *in vivo* rate of colonic mucin degradation by bacterial enzymes depends on the amount and specificity of enzymes produced and the chemical structure of the colonic mucin substrates. Homeostasis is achieved by balanced *de novo* synthesis and secretion of viable mucin from the mucosal cells.

We describe here the relationship of sialidase, sialate *O*-acetyl esterase and glycosulfatase activities present in human faecal extracts to the amount of *O*-acetylsialic acid and *O*-sulfate ester present in these molecules. Furthermore, we have detected changes in mucin composition and bacterial enzymes in ulcerative colitis (UC) which underline the importance of these interactions for the normal function of the supramucosal layer.

Materials and methods

Reagents

Cell culture products were purchased from Gibco Ltd, Edinburgh, Scotland. Sepharose CL 4B, CL 2B and Hysafe 3 were products of Pharmacia/LKB, Milton Keynes, UK. Dowex AG1 × 2 (minus 400 mesh), Bio-Gel P-6, piperazine diacrylamide and Silver stain kit were from Bio-Rad, Hemel Hempstead, UK. Silica gel 60 and Cellulose thin-layer plates were products of Merck (BDH, Poole, UK). Acetic acid detection kit was a product of the Boehringer Corporation, Lewes, Sussex, UK. [6-^{3}H]Glucosamine (740 GBq mmol^{-1}), sodium [^{35}S]sulfate (2.04 GBq mmol^{-1}), [^{14}C]-glucosamine (8.73 GBq mmol^{-1}), *N*-acetyl-[4,5,6,7,8,9-^{14}C]neuraminic acid (8.95 GBq mmol^{-1}), sodium [^{3}H]borohydride (266.4 GBq mmol^{-1}) and ^{3}H-Hyperfilm were obtained from Amersham International, Amersham, UK. Fetuin, *Clostridium perfringens* sialidase type V and *p*-nitrophenyl-β-D-galactoside were obtained from the Sigma Chemical Co., Poole, UK. *Vibrio cholerae* and influenza virus sialidases were from Calbiochem (Cambridge Bioscience, Cambridge, UK). *Arthrobacter ureafaciens* sialidase was from the Nakarai Chemical Co., Kyoto, Japan.

Oligosaccharides

The purity of all oligosaccharide substrates was checked by thin-layer and ion-exchange chromatography and the composition by gas chromatography [19–21].

N-*Acetylneuraminyl* - α(2 - 3) - *lactit* - [^{3}*H*]*ol* (1.02 GBq mmol^{-1}) and *N* - acetylneuraminyl - α(2 - 6) - lactit - [^{3}H]ol (1.15 GBq mmol^{-1}) were prepared by borotritide reduction [19] of the trisaccharides prepared from bovine colostrum [20].

N - *Acetylneuraminyl* - α(2 - 6) - N - *acetylgalacto* - *saminit* - [^{3}*H*]*ol* (2.92 MBq mmol^{-1}) was prepared by alkaline borotritide reduction of porcine seminal gel glycopeptide as described [11]. Unlabelled disaccharide was prepared in the same way using alkaline borohydride.

N-*Acetylneuraminyl*-α(2-3)-*galactosyl*-β(1-4)-[N-*acetylneuraminyl* - α(2 - 6)] - N - *acetylgalactosaminit* - [^{3}*H*]*ol* (9.0 MBq mmol^{-1}) was prepared from porcine seminal gel glycopeptide [11]. Unlabelled material was prepared in the same way using alkaline borohydride.

Galactosyl-β(1-4)-[N-*acetylneuraminyl*-α(2-6)]-N -*acetylgalactosaminit*-[^{3}*H*]*ol* (9.0 MBq mmol^{-1}) was prepared from porcine seminal gel tetrasaccharide by digestion with influenza virus sialidase. Incubations contained 3–5 μmol tetrasaccharide and 50 units of influenza virus sialidase in 50 mM sodium acetate buffer, pH 6.0, in a final volume of 15 ml at 37 °C. After 6 h the reaction was stopped by adding 10 ml ice cold water, and the sample loaded on a column (1.2 cm × 10 cm) of Dowex AG1 (2×; minus 400 mesh, acetate form) and eluted with a linear gradient of 2–350 mM pyridine acetate, pH 5.0. The purity of the product and the progress of the sialidase digestion were monitored by thin-layer chromatography on silica gel plates [20].

N-*Acetylneuraminyl*-α(2-3)-*galactosyl*-β(1-4)-N-*acetylgalactosaminit*-[^{3}*H*]*ol* (5 MBq mmol^{-1}) and the un labelled trisaccharide were isolated from bovine fetuin by alkaline borotritide/borohydride treatment, respectively, using the same methods as described above [11].

Lactitol 6 - O - *sulfate* [*galactosyl* - *6* - O - *sulfate* - β(*1* - *4*) - *glucitol*]. Lactose 6-*O*-sulfate [galactosyl-6-*O*-sulfate-β(1-4)glucose] was prepared chemically [22] and used to synthesize lactitol 6-*O*-sulfate [galactosyl-6-*O*-sulfate-β(1-4)-glucitol] by reduction with a 25-fold molar excess of sodium borohydride for 2 h at pH 9 in 0.1 M Na_2CO_3 at 20 °C. A radioactive preparation of lactit-[^{3}H]ol 6-*O*-sulfate [galactosyl-6-*O*-sulfate-β(1-4)-glucit-[^{3}H]ol] using sodium [^{3}H]borohybride in the reduction step yielded a product with a specific radioactivity of 0.64 GBq mmol^{-1}.

Glycoproteins

Bovine submandibular gland glycoprotein (BSM) was prepared as described [19]. The sialate-*O*-acetyl groups of BSM are located on the hydroxyls of carbons 7, 8, or 9 as mono- and di-*O*-acetyl esters [23]. The preparation used contained 1.4 μmol *O*-acetyl ester per mg dry weight (2.1 mol *O*-acetyl ester per mol sialic acid). Saponification was carried out at a glycoprotein concentration of 1 mM with respect to sialic

acid in 0.1 M NaOH for 45 min at 4 °C. The solution was neutralized and dialysed against an excess of water before lyophilization. Radiolabelling of sialic acids in saponified BSM was by mild periodate oxidation and borotritide reduction as described before [19], yielding a product with specific radioactivity of 1.22 GBq $mmol^{-1}$ sialic acid.

Human α_1-acid glycoprotein was obtained from the Scottish National Blood Transfusion Service, Edinburgh, Scotland [19]. Radiolabelling of sialic acids in α_1-acid glycoprotein was as described before [19], yielding a product with a specific radioactivity of 1.28 GBq $mmol^{-1}$ sialic acid. Asialo-α_1-acid glycoprotein was prepared by treatment with 0.1 M HCl for 60 min at 80 °C followed by dialysis and lyophilization. The product contained 0.23 μmol terminal galactose per mg dry weight. This galactose is in β(1-4) linkage to N-linked oligosaccharides [24]. Terminal non-reducing galactose residues in the asialoglycoprotein were tritiated using galactose oxidase/sodium borotritide [25], yielding a tritiated glycoprotein product with 0.34 GBq per mmol galactose.

Antifreeze glycoprotein was a gift of Dr A. DeVries, University of Illinois, USA. The galactose content of the glycoprotein was 1.14 μmol per mg dry weight. All of the galactose is contained in the form of the disaccharide Galβ(1-3)GalNAc- linked to threonine [24, 26]. The glycoprotein was labelled in the galactose residues by the galactose oxidase/borotritide method [25], resulting in a product with a specific radioactivity of 1.49 GBq per mmol galactose.

Partial purification of faecal extracts

Faecal extracts were prepared as before [11] and 5 ml portions were taken and made up to 80% saturation with ammonium sulfate before stirring overnight at 4 °C. The pellet (approx. 70 mg protein) was collected by centrifugation at 35000 × **g** for 20 min at 4 °C, dissolved in 1 ml 100 mM triethanolamine buffer, pH 7.8, and applied to a column (30 cm × 1 cm) of Sephadex G-100 equilibrated in the same buffer. Fractions of 1 ml were collected, and the OD at 280 nm monitored. The peak eluting at fractions 6–11 contained all enzyme activities, and was concentrated by precipitation with 80% ammonium sulfate as above. The pellet was dissolved in 1 ml ethanolamine buffer and dialysed for 2 h against three changes of 1 l of the same buffer. The volume was measured and the fraction used for enzyme assays. Recovery of activity as judged by total sialidase, and arylesterase activity was $85 \pm 7\%$ and $93 \pm 5\%$ ($n = 12$), respectively, and yielded the highest recovery compared with alternative methods of concentrating the enzyme activities from the faecal extracts.

Enzyme assays

The chosen conditions were optimal with respect to enzyme concentration, incubation time, pH and substrate concentration [10, 11, 19]. They are expressed relative to the dry weight of the faecal samples [11]. One unit of activity is defined as release of 1 μmol of product per min.

Sialidase (E.C. 3.2.1.18) was assayed as before [10, 11] for both ^{3}H-oligosaccharide and ^{3}H-glycoprotein substrates diluted with unlabelled substrate to give a final concentration of 2.5 mM or 1 mM relative to sialic acid for oligosaccharide and glycoprotein substrates, respectively. The incubation contained 50 μl faecal extract in a final volume of 100 μl. The minimal product level detectable with this assay was 1.6 nmol for *N*-acetylneuraminyl-α(2-6)-*N*-acetylgalactosaminit-[^{3}H]ol, 4.8 nmol for galactosyl-β(1-4)-[*N*-acetylneuraminyl-α(2-6)]-*N*-acetylgalactosaminit-[^{3}H]ol, 3.0 nmol for *N*-acetylneuraminyl-α(2-3)-galactosyl-β(1-4)-*N*-acetylgalactosaminit-[^{3}H]ol, 2.1 pmol for *N*-acetylneuraminyl-α(2-3)-lactit-[^{3}H]ol, 2.2 pmol for α_1-acid glycoprotein, and 5.2 pmol for BSM.

Action of sialidases on O-*acetylated and saponified BSM* was measured in triplicate in a total volume of 800 μl containing saponified or native (nonsaponified) glycoprotein that was 1 mM with respect to sialic acid, 100 mM sodium acetate at pH 5.5 and enzyme fraction. Incubation was at 37 °C for various times. Aliquots of 80 μl were removed and mixed with 20 μl of 0.5 M NaOH and kept for 30 min at 4 °C. The sample was acidified by the addition of 25 μl 0.5 M HCl and free sialic acid determined by the periodate–thiobarbituric acid assay [27].

Sialate O-*acetylesterase* (E.C. 3.1.1.53) was measured as before [10, 11] in incubation mixtures containing 100 μl faecal extract in a final volume of 200 μl. The supernate was assayed for acetic acid using a commercial kit from the Boehringer Corporation, Lewes, Sussex, UK. The lowest level of product detectable with this assay system was 15 nmol.

N-*Acetylneuraminate lyase* (E.C. 4.1.3.3) was measured as before [19]. The lowest detectable level of product in this assay was 20 pmol.

Glycosulfatase (E.C. 3.1.6.3) was assayed using lactit-[^{3}H]ol 6-sulfate [galactosyl-6-*O*-sulfate-β(1-4)-glucit-[^{3}H]ol] [15]. Incubation mixtures contained 50 μl enzyme extract and 8 kBq lactit-[^{3}H]ol sulfate in 0.1 M sodium acetate buffer, pH 5.0, in a final volume of 100 μl. Incubation was for 60 min at 37 °C. The reaction was stopped by the addition of 1 ml 95% ethanol and the mixture centrifuged at 12000 × **g** for 2 min. The supernate was applied to a 1 ml column of Dowex 1 × 8 formate resin and washed with two 5 ml aliquots of distilled water. After drying the unabsorbed fraction at 80 °C the residue was redissolved in 200 μl water and mixed with 10 ml scintillation fluid before counting. The lowest level of product detectable by this method was 2.6 pmol.

β-Galactosidase (E.C. 3.2.1.23) was assayed with *p*-nitrophenyl-β-D-galactoside as described before, with a lowest level of detection of 2 nmol [10, 11].

The activity against Galβ(1-3)-linked galactose was tested in assay mixtures containing 3.3 kBq ^{3}H-antifreeze glycoprotein, 0.04 mg unlabelled glycoprotein (0.046 µmol bound galactose) and 25 µl faecal extract in 0.2 M sodium acetate buffer, pH 6.0, in a final volume of 100 µl. Incubation was for 30 min at 37 °C. The reaction was stopped by addition of 1 ml ice cold water and the sample was loaded on a column (100 cm × 1 cm) of Sephadex G-25 superfine equilibrated in 0.1 M sodium bicarbonate. The peak eluting in the position of free [^{3}H]galactose was collected and measured. Blanks contained faecal extract heated at 95 °C for 5 min. The lowest level of activity detectable with this assay was 1.1 pmol.

The activity against Galβ(1-4) was assayed in 0.2 M sodium acetate, pH 6.0, containing 3.9 kBq ^{3}H-asialo-α_1-acid glycoprotein, 0.34 mg unlabelled glycoprotein (0.8 µmol bound galactose) and 25 µl faecal extract in a final volume of 100 µl. Incubation was for 40 min at 37 °C and was stopped by the addition of 500 µl trichloroacetic acid/phosphotungstic acid (15%/5% by vol). After centrifugation at 12000 × **g** for 2 min, a 500 µl portion of the supernatant was removed and its radioactivity determined. Blanks included faecal extract heated at 95 °C for 5 min. The lowest level of activity detectable in this assay was 4.9 pmol.

Arylesterase (E.C. 3.1.1.3) was assayed as before [10, 11]. The minimal amount of product detectable in this assay was 2 nmol.

Proteinase activity was measured using azocasein [11].

Organ culture and mucin isolation

Organ culture was carried out as described [28]. Briefly, mucosal tissue obtained from surgery was cut into squares of approximately 3 mm^2 and up to six were placed with the mucosal (luminal) surface uppermost on lens tissue over stainless steel grids in plastic culture dishes with a central well containing 2 ml medium. Radioactive precursors 370 kBq [^{3}H]glucosamine and 925 kBq sodium [^{35}S]-sulfate or 185 kBq [^{14}C]glucosamine for each dish were added to the medium immediately before incubation, and culture was for 24 h at 37 °C in oxygen/CO_2 (95%/5%). After incubation the secreted fraction was collected [28]. The secreted material was fractionated on columns (30 cm × 1 cm) of Sepharose CL 4B equilibrated in 10 mM Tris-HCl, pH 8.0, and eluted with the same buffer. The high molecular weight peak eluting at the excluded volume (Vo, fractions 5–7) was quantified and taken for further analysis.

Digestion of labelled secreted colonic mucins by faecal extracts

The material eluting at the Vo of Sepharose CL 4B columns (fractions 5–7) was pooled and freeze dried. Aliquots of this material were incubated with faecal extracts from normal or UC patients. The mucin (5000–50000 disintegrations min^{-1} as ^{35}S or ^{3}H) was incubated for 24 h in phosphate-buffered saline (PBS; 0.01 M sodium phosphate buffer, pH 7.0, in 0.15 M NaCl) at 37 °C. Incubations contained labelled mucin and 0.5 ml faecal extract in a final volume of 1 ml. Incubations were stopped at different times on ice, applied to Sepharose CL 2B columns, and eluted as described above in PBS. The proportion of the excluded mucin fraction appearing as low molecular weight material after incubation with faecal extract (indicated in Fig. 6) was expressed as a percentage of the total isotope (^{35}S or ^{3}H) in each incubation and corrected for the amount of faecal extract on a dry weight basis [11]. Normal (two) and UC (two) mucin fractions were tested with three normal and three UC faecal extracts.

Removal and separation of sialic acids

Sialic acids in ^{14}C radiolabelled and nonlabelled colonic mucins were released by hydrolysis in 1 ml 2 M acetic acid at 80 °C for 4 h. The sialic acids isolated after dialysis were purified over Dowex 50-W (H^+) and AG3 × 4 formate form as described [29]. The purified sialic acids were separated by cellulose thin-layer chromatography [27] together with a carrier mixture of authentic *O*-acetyl-*N*-acetylneuraminic acids prepared by partial saponification of penta-acetylneuraminic acid. The mono-, di- and tri-*O*-acetylated derivatives were identified by comparison with the *O*-acetyl sialic acid standards and quantified after scraping the cellulose layer from the plates. Standard samples were visualized on the plates with the orcinol spray [27].

SDS-polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis on 3% gels using piperazine diacrylamide in place of bisacrylamide was carried out as before [30]. Samples were run on 3% separating gels with 2.5% stacking gels at a constant voltage of 70 V per gel for approx. 4 h. Molecular weight markers of myosin (205000) and IgM (990000) were used and detected by silver staining (Bio-Rad). The gels were air dried on Whatman 3M paper and autoradiography carried out using ^{3}H-Hyperfilm for periods up to 3 weeks.

Other methods

Radioactivity was measured in an LS 1510 liquid scintillation counter (LKB Wallac) using Hysafe 3 scintillation fluid with programmes for single and double label detection, and correction for quench with external standard calibration and for ^{35}S decay. Statistical analysis of the disease groups was carried out using the Mann-Whitney U test. Thin-layer chromatographic separation of sialic acids [27] and oligosaccharides [20] was as described.

Results and discussion

Sialidase activity in faecal extracts

Previous work has demonstrated the presence of sialidase and sialate *O*-acetyl esterase in rat [31] and human [11]

Table 1. Action of sialidases on oligosaccharide and glycoprotein substrates.[a]

Substrates	*Sialidase activity (Mean ± SD)*				
	*Faecal**	*AU***	*VC***	*CP***	*IV***
Neu5Acα(2-3)lactitol	100 (0.47 ± 0.1)	100 (39.4 ± 7.1)	100 (9.7 ± 0.5)	100 (20.6 ± 3.2)	100 (4.7 ± 0.6)
Neu5Acα(2-6)lactitol	65 ± 12	172 ± 34	45 ± 11	40 ± 7	<1
Neu5Acα(2-6)GalNAc-ol	640 ± 120	159 ± 25	29 ± 4	43 ± 12	<1
Neu5Acα(2-3)Galβ(1-3)GalNAc-ol	1290 ± 295	110 ± 31	210 ± 45	160 ± 34	74 ± 12
Galβ(1-3)[Neu5Acα(2-6)]GalNAc-ol	710 ± 225	143 ± 27	95 ± 18	80 ± 20	<1
α_1-Acid glycoprotein	31 ± 9	60 ± 12	979 ± 278	540 ± 175	290 ± 78
Bovine salivary mucin	75 ± 15	54 ± 9	385 ± 110	127 ± 28	<1

[a] Incubations were carried out as described in the Materials and methods section. The activity is the mean ± SD of the initial rate of reaction expressed as a percentage of that found for Neu5Acα(2-3)lactitol (100%). The figures in parentheses are the measured rates for each sialidase preparation as *, μmol sialic acid released per min per g dry weight or **, μmol sialic acid released per mg protein. AU, *Arthrobacter ureafaciens*; VC, *Vibrio cholerae*; CP, *Clostridium perfringens*; IV, influenza virus.

faeces and their widespread occurrence in the main groups of human enteric bacteria [10]. The action of sialidase on different oligosaccharide and glycoprotein substrates is shown in Table 1 for normal faecal extracts. Faecal extract shows good activity against mucin related substrates, particularly the oligosaccharide substrates which are hydrolysed 6–12 times better than sialyl-α(2-3)-lactitol. An apparent K_m of 1.67 ± 0.61 mM and a V_{max} of 0.41 ± 0.07 U per g dry weight was found (mean ± SD, $n = 3$) with the disaccharide *N*-acetylneuraminyl-α(2-6)-*N*-acetylgalactosaminit-[^{3}H]ol. Comparison confirms the general α(2-3) preference described for the other sialidases and the slight α(2-6) preference for the *Arthrobacter ureafaciens* enzyme [32]. The influence of oligosaccharide structure on specificity is shown for the *Vibrio cholerae* and *Clostridium perfringens* enzymes, where the α(2-6) substrate Galβ(1-3)[Neu5Acα(2-6)]GalNAc-ol is cleaved better than the disaccharide Neu5Acα(2-6)GalNAc-ol. Further direct evidence for the α(2-3) preference in faecal extract sialidase activity is shown in the digestion of Neu5Acα(2-3)Galβ(1-3)[Neu5Acα(2-6)]-GalNAc-ol where the trisaccharide Galβ(1-3)[Neu5Acα(2-6)]GalNAc-ol was found to be the final product of digestion, on thin-layer chromatography (Fig. 1). The marked α(2-3) specificity of the influenza virus sialidase [12] is apparent from both kinetic (Table 1) and thin-layer (Fig. 1) experiments. The high activity with oligosaccharide substrates relative to glycoprotein substrates raises the possibility that the release of sialyl oligosaccharides by an *O*-glycanase may be an important step in mucin degradation.

Inhibition of faecal sialidase activity

Table 2 shows the action of three known sialidase inhibitors [Neu5Ac2en, *N*-(4-nitrophenyl)oxamic acid and Cu^{2+}] and the product of sialidase action (Neu5Ac) with α_1-acid glycoprotein, BSM, Neu5Acα(2-3)lactitol and Neu5Acα(2-6)GalNAc-ol as substrates. Strong inhibition (>70%) was found for Neu5Ac2en and Cu^{2+} at 2.0 mM for all substrates, while only weak inhibition (<10%) was detected with *N*-(4-nitrophenyl)oxamic acid and Neu5Ac (Table 2). The lack of inhibition with *N*-(4-nitrophenyl)oxamic acid is unusual for bacterial sialidases [12], and is a novel property of sialidase in enteric bacteria.

Influence of substrate O-acetylation on faecal sialidase activity

Assay of the total sialidase activity with native and saponified BSM showed a significantly greater rate of sialic acid release with the *O*-acetyl ester free substrate (Fig. 2). Similar results were obtained with two other faecal extracts. The sialic acids hydrolysed from native BSM by the faecal extract comprised Neu5Ac, Neu5Gc and their mono-*O*-acetylated derivatives. No di-*O*-acetylated sialic acids were

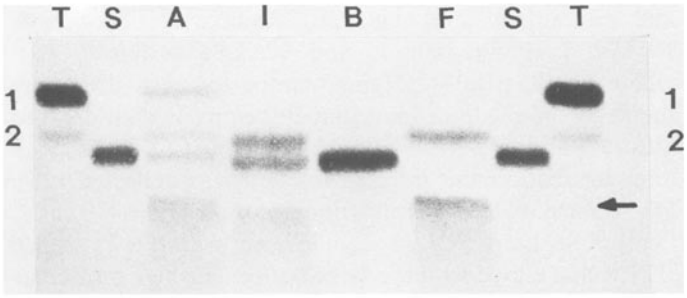

Figure 1. The action of sialidases on the disialyltetrasaccharide Neu5Acα(2-3)Galβ(1-3)[Neu5Acα(2-6)]GalNAc-ol. Silica gel thin-layer chromatographic separation of the reaction products of partial acid hydrolysis (A), influenza virus (I) and faecal (F) sialidase and buffer alone (B) on the substrate Neu5Acα(2-3)Galβ(1-3)[Neu5Acα(2-6)]GalNAc-ol (S). A mixture (T) of the trisaccharides Neu5Acα(2-3)Galβ(1-3)GalNAc-ol (1) and Galβ(1-3)[Neu5Acα(2-6)]GalNAc-ol (2) were run on the same thin-layer plate. The position of free sialic acid liberated during the reaction is shown by the arrow.

Table 2. Inhibition of sialidase activity in faecal extracts.[a]

	% inhibition (Mean ± SD)			
	α_1-Acid glycoprotein	*Neu5Acα(2-3)-lactitol*	*Bovine salivary glycoprotein*	*Neu5Acα(2-6)-GalNAc-ol*
Neu5Ac2en				
0.1 mM	25 ± 7	85 ± 8	77 ± 4	81 ± 9
2.0 mM	79 ± 3	89 ± 7	92 ± 3	91 ± 8
N-(4-nitrophenyl) oxamic acid				
0.1 mM	6 ± 3	9 ± 8	10 ± 2	13 ± 8
2.0 mM	10 ± 7	15 ± 7	11 ± 3	11 ± 7
Cu^{2+}				
0.1 mM	21 ± 3	78 ± 6	69 ± 4	74 ± 11
2.0 mM	72 ± 7	81 ± 8	78 ± 5	79 ± 9
Neu5Ac				
0.1 mM	1 ± 3	7 ± 6	5 ± 4	1 ± 3
2.0 mM	5 ± 4	9 ± 7	4 ± 3	3 ± 4

[a] Inhibition was measured at different concentrations of each inhibitor under assay conditions as described for each substrate in Table 1. Activity in the absence of inhibitor was 100%. The results are the mean ± SD of three different incubations with two normal faecal extract samples.

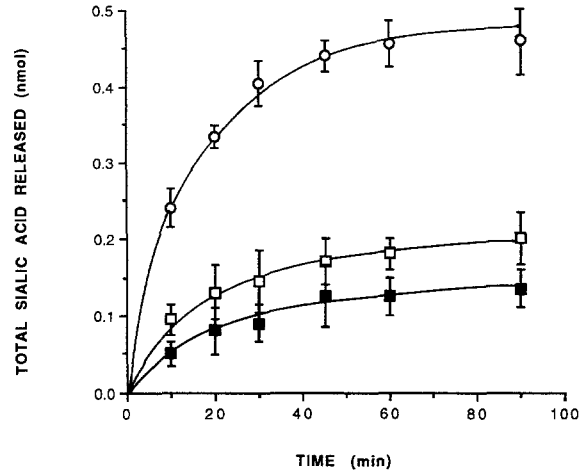

Figure 2. The action of faecal extract sialidase on submandibular gland glycoprotein. Normal faecal extract was incubated with native bovine submandibular gland glycoprotein (□) or with saponified glycoprotein (○) at the same final concentration of sialic acid. Total released sialic acid was measured. Native glycoprotein was also incubated under the same conditions in the presence of 1 mM PMSF (■). The error bars show the variation in triplicate incubations with the same normal faecal extract.

detected on cellulose thin-layer chromatography (data not shown). The reduction in the rate of sialidase activity with di-*O*-acetyl sialic acids is directly related to the presence of sialic acids with 2 or 3 *O*-acetyl esters in colonic mucins (see below [4–7]). These results imply a role for the sialate *O*-acetyl esterase in regulating the overall rate of sialic acid release through enteric bacterial sialidase.

Sialate O-acetyl esterase activity in faecal extracts

The activity of sialate *O*-acetyl esterase could be measured in faecal extracts only after fractionation on Sephadex G-100, as the presence of pigments and other compounds interferes with the acetic acid detection kit used in the assay for the enzyme [11]. The pH optimum was determined at 7.8 and an apparent K_m of 1.2 ± 0.3 mM for *O*-acetyl sialic acids in BSM and a V_{max} of 35 ± 12 mU per g dry weight (mean ± SD, $n = 3$) were measured. Assays of a normal faecal extract in the presence of the esterase inhibitor phenylmethylsulfonyl fluoride (PMSF) at 1 mM resulted in a reduction of activity of 45 ± 6% ($n = 3$). Preincubation of the faecal extract with 1 mM PMSF gave a reduction in the rate of sialic acid release from native BSM (Fig. 2) indicating the inhibition of sialate *O*-acetylesterase activity and significant action of the esterase in releasing *O*-acetylated sialic acids for sialidase hydrolysis. The sialate *O*-acetyl esterase activity in 22 normal faecal extracts was over 100-fold lower than that of sialidase measured in the same samples (Table 3). The lower activity of the esterase suggests that, although this enzyme may release the block on sialidase action caused by the di-*O*-acetyl and tri-*O*-acetyl-sialic acids in colonic mucins, it does so at a slow rate and this is in keeping with the regulatory role in mucin degradation proposed earlier [11].

Glycosulfatase activity in faecal extracts

Oligosaccharide sulfation is thought to be related to the rate of mucin degradation [15–17, 33]. The enzymes responsible for the removal of these carbohydrate *O*-sulfate esters have been demonstrated in human faecal extracts [15, 17] and in different enteric bacteria [10, 16, 17, 33] using different substrates. Glycosulfatase activity was detected in faecal extracts using the substrate lactit-[^{3}H]ol 6-sulfate. The activity showed a major pH optimum at 5.0, with a shoulder at pH 7.0 and no activity at pH 3.5 or 9.0. Determination of the apparent K_m yielded a value of 0.8 ± 0.2 mM for lactit-[^{3}H]ol 6-sulfate and a V_{max} of 3.8 ± 0.7 mU per g dry wt (mean ± SD, $n = 3$). The activity was partially dependent on divalent cations as 70%

Table 3. Activity of sialidase, sialate *O*-acetyl esterase and glycosulfatase in normal faecal extracts.[a]

	Total activity (μmole h^{-1} mg^{-1})	K_m (mM)	V_{max} (μmole h^{-1} mg^{-1})
Sialidase	15.9 ± 4.0	1.67 ± 0.55	24.6 ± 6.6
Sialiate *O*-acetyl esterase	0.15 ± 0.07	1.20 ± 0.30	0.35 ± 0.12
Glycosulfatase	1.12 ± 0.54[b]	1.00 ± 0.42	2.50 ± 0.70[b]
n	22	3	3

[a] The activities in faecal extracts were measured as described in the Materials and methods section and are expressed as the mean ± SD, activity per mg dry weight of faecal suspension.
[b] Activity is nmole h^{-1} mg^{-1}.

inhibition was found at 25 mM EDTA and this could be restored fully by either Ca^{2+} or Mg^{2+} but not by Na^+ or K^+. The lactit-[^{3}H]ol 6-sulfate substrate may detect only part of the total faecal glycosulfatase activity, and additional defined substrates are required to detect the existence of enzymes specific for 3-*O*- and 4-*O*-ester sulfate groups on galactosyl and *N*-acetylglucosaminyl residues in mucins.

Faecal extract enzyme activities in ulcerative colitis

The faecal extract enzyme activities, including those related to the degradation of colonic mucins, were assayed in 22 normal subjects and 15 established ulcerative colitis patients (Table 4). The activities of sialidase with Neu5Acα-(2-6)GalNAc-[^{3}H]ol, β-galactosidase with *p*-nitrophenyl galactoside, galactose β(1-3)- and β(1-4)-linked substrates and that of arylesterase were unchanged between the groups. However, the sialate *O*-acetyl esterase, glycosulfatase acylneuraminate lyase and proteinase activities were all significantly higher in the ulcerative colitis group. The elevation in enzymatic activity releasing sialic acid *O*-acetyl esters and carbohydrate *O*-esters in disease underlines the importance of these peripheral groups in mucin degradation.

Table 4. Activity of mucin degrading enzymes in normal and UC faecal extracts.[a]

Enzyme	*Activity* (μmole h^{-1} mg^{-1})		
	Normal	*Colitis*	*p*
Sialidase	15.9 ± 4.0	15.7 ± 4.8	NS
Sialate *O*-acetyl esterase	0.15 ± 0.07	0.39 ± 0.13	<0.02
Glycosulfatase*	1.12 ± 0.54	7.0 ± 2.6	<0.01
Acylneuraminate pyruvate lyase**	37.4 ± 8.4	214 ± 42	<0.05
β-Galactosidase *p*-nitrophenyl galactoside	56.7 ± 10.3	63.3 ± 17.7	NS
β(1-3)galactoside	8.6 ± 3.1	11.2 ± 2.9	NS
β(1-4)galactoside	133.9 ± 21.3	113.5 ± 29.3	NS
Arylesterase	32.8 ± 3.5	23.7 ± 13.9	NS
Proteinase$^+$	51.6 ± 14.2	108.5 ± 25.3	<0.01
Dry weight (mg ml^{-1})	40.1 ± 1.8	38.8 ± 2.8	NS
n	22	15	

[a] Activities were assayed as described in the Materials and methods section and are expressed as the mean ± SD, μmol release of product per h relative to the dry weight of faecal suspension. Exceptions are *, nmol h^{-1} mg^{-1}, **, pmol h^{-1} mg^{-1}, and $^+$OD units h^{-1} mg^{-1} [11]. NS = not significant.

Sialylation and sulfation of colonic mucins

Secreted mucin from metabolically labelled tissue could be isolated on Sepharose CL 4B. The mucin separated in this way from normal and UC patients gave the profiles shown in Fig. 3(a, b). In five normal incubations, the mucin rich peak (fractions 5–7) showed a $^{35}S/^3H$ ratio of 1.25 ± 0.45 (mean ± SD). In UC patients, the mucin had a $^{35}S/^3H$ ratio of 0.23 ± 0.11 (mean ± SD, $n = 6$). The elution profile for [^{14}C]glucosamine labelling was the same as for the ^{3}H labelled precursor. Autoradiography of the mucin-rich peak separated on SDS-PAGE showed only high molecular weight material in excess of 10^6 for both normal and UC patients (Fig. 4). The reduction in mucin sulfation associated with UC is in agreement with earlier histological results [7] and biochemical data [34, 35].

Sialic acid O-acetylation of colonic mucins

Separation of individual sialic acids released from [^{14}C]-glucosamine labelled mucin by mild acid hydrolysis showed

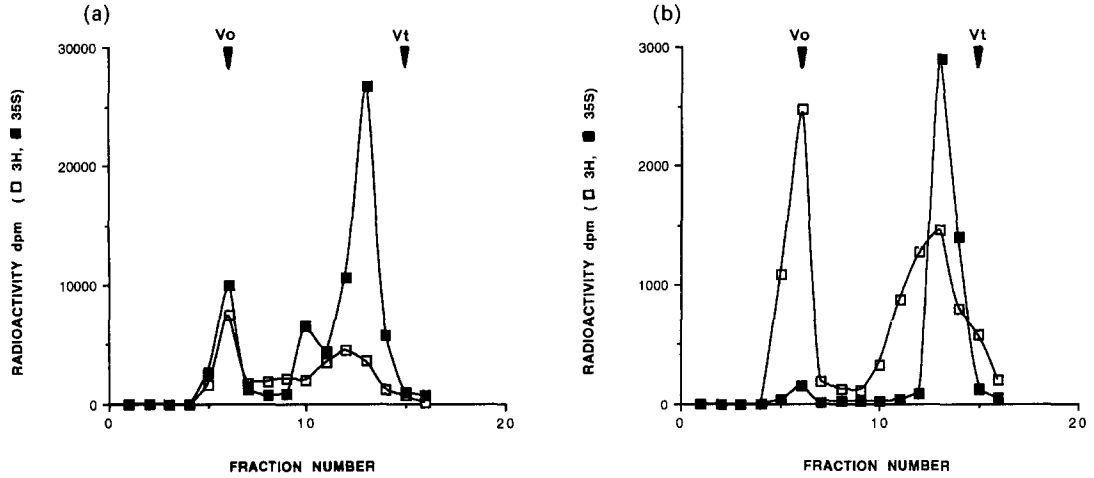

Figure 3. Metabolic labelling of normal and UC colonic mucins. Colonic biopsies from normal (a) and UC (b) patients were cultured under standard conditions described in the Materials and methods section. The secreted (medium) fraction was dialysed and applied to a column of Sepharose CL-4B equilibrated and eluted with 0.1 M Tris-HCl, pH 8.0, 2 ml fractions were collected and the radioactivity measured. The excluded (Vo) and included (Vt) limits of the column are shown. Fractions 5–7 were pooled amd used in subsequent experiments. Note the tenfold difference in the radioactivity scale on the normal compared with the colitis samples.

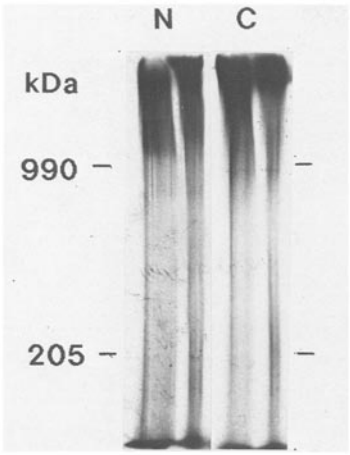

Figure 4. Autoradiographic analysis of colonic mucin separated by SDS-polyacrylamide gel electrophoresis on 3% gels. The Vo mucin peak (fractions 5–7 in Fig. 3) was isolated from two normal (N) and two colitic (C) patients. The samples were run on 3% polyacrylamide gels as described in the Materials and methods section. The gels were air dried and submitted to autoradiography. Molecular weight markers of IgM (990 000) and myosin (205 000) were run on the same gel and stained with silver stain.

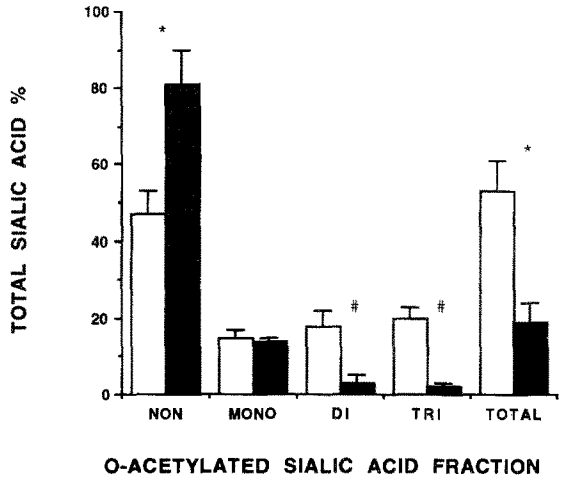

Figure 5. Sialic acid *O*-acetylation in normal and colitic colonic mucins. The secreted (medium) fraction from organ cultures labelled with [^{14}C]glucosamine was fractionated on Sepharose CL-4B, the Vo (fractions 5–7) peak collected and the sialic acids removed and separated by thin-layer chromatography as described in the Materials and methods section. The radioactivity migrating with non-, mono-, di- and tri-*O*-acetyl-*N*-acetylneuraminic acids was measured, and is expressed as a percentage of the total sialic acid radioactivity. The total *O*-acetylated sialic acid fraction is also shown. The significance of the normal (□) versus colitis (■) results was tested using the Mann-Whitney U test for five normal and six colitis patients. Significance is shown as *, $p < 0.005$; #, $p < 0.001$.

that over 50% of all sialic acids are *O*-acetylated in normal mucin, and that approx. 40% are di-*O*- or tri-*O*-acetylated (Fig. 5). In contrast, a significant reduction in the di-*O*-and tri-*O*- acetylated derivatives is seen in UC, comparing six patients with five normal individuals. This result is in good agreement with histochemical studies [5, 7] on UC and biochemical studies on colorectal cancer [4–6, 36].

Digestion of normal and UC mucins by faecal extracts

The isolated radiolabelled mucins are useful substrates to assess the rate of mucin degradation. The digestion of $^{35}S/^{3}H$ dual labelled mucin with faecal extract resulted typically in the cleavage of the high molecular weight peak seen on Sepharose CL 4B and SDS-PAGE into intermediate and low molecular weight components (Fig. 6), indicating the action of proteinases or depolymerizing enzymes in addition to glycosidases and sulfatases. The differences found in the sulfation and *O*-acetylation patterns for UC mucins (Figs 3, 5) were reflected in their behaviour with normal and UC faecal extracts.

The rates of digestion of normal and UC colonic mucins by normal and UC faecal extracts are shown in Fig. 7. Normal mucin was degraded more rapidly by the UC compared with the normal faecal extract for both ^{35}S and ^{3}H labels. The UC mucins were digested at a greater rate than the normal mucins tested, but the difference in action between the normal and UC faecal extracts was not as marked. Two normal and two UC mucin fractions were tested, each with three normal and three UC faecal extracts: similar results to those shown in Fig. 7 were found.

The results obtained with colonic mucins and faecal extracts illustrate the physiological importance of *O*-acetylated sialic acids and sulfate in normal mucin structure. They also provide direct evidence of their involvement in mucin defects arising in UC [7, 8, 11, 17, 33].

Conclusion

The rate of mucin degradation by bacterial enzymes in the colon depends on the efficiency of removal of the terminal nonreducing residues on the mucin oligosaccharide chains. The enzymes responsible for the removal of these terminal residues are often found at low levels relative to the glycosidases degrading the remaining oligosaccharide structures [1, 11]. Regulation of the removal of these peripheral structures will thus govern the rate of mucin degradation.

The relationship between human colonic mucin sialylation, *O*-acetylation and sulfation and their enzymatic removal is not yet understood, because a complete structural analysis is not available for the oligosaccharides [1, 3, 6, 17]. However, the nature and relative amounts of sialidase, sialate *O*-acetyl esterase and glycosulfatase activities in faecal extracts together with the *O*-acetyl-sialic acid and sulfate content of metabolically labelled colonic mucins reported in this study support the hypothesis that these peripheral structures are directly involved in the regulation of mucin degradation [1, 8, 11, 16, 17, 33].

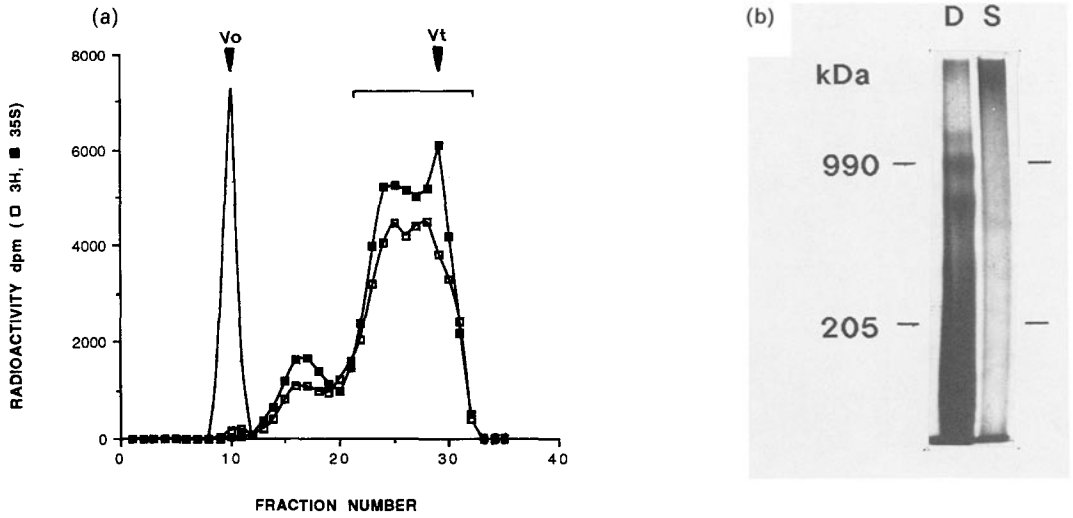

Figure 6. Degradation of human colonic mucin by faecal extract. (a) Normal colonic mucin prepared and isolated as described in the Materials and methods section and comprising fractions 5–7 as shown in Fig. 3(a) was rechromatographed on Sepharose CL 2B, and the peak at the Vo (solid line only) used as substrate. The products of incubation with UC faecal extract are shown for [^{3}H]glucosamine (□) and [^{35}S]sulfate (■) label. The bar indicates the fraction of low molecular weight material used to calculate degradation in the time dependent digestion studies shown in Fig. 7. (b) Aliquots of the starting material (S) and 24 h digested product (D) were run on SDS-PAGE in 3% gels and submitted to autoradiography. The fragmentation of the high molecular weight material is seen on both analytical techniques.

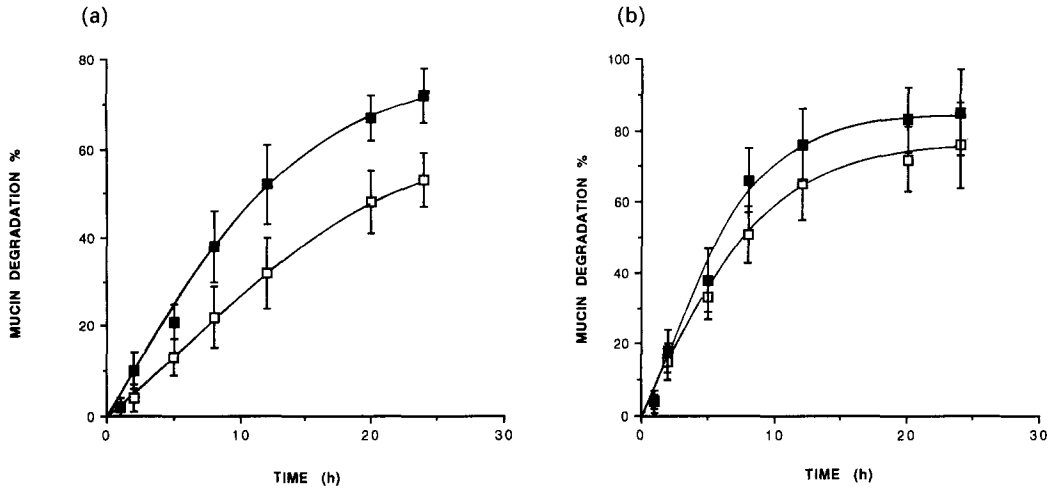

Figure 7. Degradation of normal and colitic mucin by faecal extracts. Individual samples of (a) normal and (b) UC secreted mucin were prepared as described in the Materials and methods section and correspond to Sepharose CL 4B fractions 5–7 as shown in Fig. 3. The mucins were incubated for different times with individual faecal extracts prepared from a normal (□) and a colitis patient (■). The amount of degraded mucin was calculated as a percentage of the starting material, and corresponds to the low molecular weight fraction indicated in Fig. 6(a). The bars show the variation between triplicate assays.

Sialidase activity in faecal extracts is significantly higher than either sialate *O*-acetyl esterase or sulfatase levels. As sialidase activity is regulated by *O*-acetylation [10, 14] and sulfation patterns, the increases in sialate *O*-acetyl esterase and sulfatase activities seen in UC will lead to an increase in the efficiency of the sialidase action and an increase in the rate of mucin degradation. No change is seen in the level of sialidase activity in UC, and this may be because sufficient enzyme is already present to increase mucin desialylation once the controlling influences of *O*-acetyl esters and sulfate are reduced or removed. In addition, the isolated UC colonic mucins show a loss of sulfate and a reduction in di-*O*- and tri-*O*-acetyl sialic acids [35, 36]. These structural changes reduce the protection against sialidase action and subsequently other glycosidases acting on the oligosaccharides, resulting in the increased rate of UC mucin degradation. The increased enzyme activities and loss of protective residues on secreted mucin both

contribute to the abnormal mucin metabolism found in UC.

The balance between the synthesis of sufficient mucin with the correct sialylation, *O*-acetylation and sulfation, and its degradation by bacteria will govern the amount present in the supramucosal layer providing the defensive and nutritional functions required at the colorectal mucosal surface.

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